The role of creatine kinase in hypertension: Therapeutic perspectives

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"Whatever you do will be insignificant, but it is very important that you do it"

Mahatma Gandhi

COLOFON

The Role of Creatine Kinase in Hypertension: Therapeutic Perspectives

PhD Thesis, University of Amsterdam, Amsterdam, the Netherlands

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The Role of Creatine Kinase in Hypertension: Therapeutic Perspectives

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aan de Universiteit van Amsterdam

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prof. dr. Ir. K.I.J. Maex

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Chapter 1

Introduction and outline of the thesis

I. Introduction

Hypertension affects approximately 25% of the adult population worldwide, and its prevalence is predicted to increase by 60% by 2025. A total of 1.56 billion people may be affected, with the increase largely in low and middle-income countries.[1,2] Globally, it is still the major risk factor for premature cardiovascular mortality.[3,4] The pathogenesis of hypertension is multifactorial, and environmental and biological circumstances contribute to the occurrence of the disease.[1-6]

Blood pressure control

The mean arterial pressure depends on the cardiac output and total peripheral resistance of the vessels, as expressed by the equation: MAP = CO x TPR, where MAP is the mean arterial pressure, CO the cardiac output, and TPR the total peripheral resistance.[6,7] In established hypertension, cardiac output is normal and a raised peripheral resistance is mostly responsible for the elevated pressure.[7] Total peripheral resistance is affected by blood viscosity, by the design and connectivity of the vascular networks, and by the diameter and length of the vessels in this network.[7] By far the greatest contribution to peripheral resistance is made by small arteries and arterioles, also referred to as resistance arteries.[8] In hypertensive patients, the increased total peripheral resistance of blood vessels originates from both structural and functional changes in the resistance vessels, including changes in the level of vasoconstriction in these vessels.[9] While the relation between MAP and TPR seems to be simple, the key question is how the various systems involved in blood pressure control contribute to the eventual development of a stable increase in both pressure and resistance in established hypertension. A wide variety of physiologic systems, which interact in a complex fashion, have been found to influence blood pressure. [6,7] Included among these systems are baroreceptors that sense acute changes in pressure in vessels; atrial natriuretic peptides produced by heart in response to increased atrial pressure; the renin-angiotensin-aldosterone system, which influences vascular volume homeostasis and renal salt handling; the adrenergic receptor system, which influences heart rate, cardiac contraction; and factors produced by blood vessels that cause vasodilation, such as nitric oxide, or contraction, such as endothelin. [6,7] Resistance arteries are characterized by the presence of myogenic tone, i.e. their intrinsic ability to contract in response to a sudden increase of transmural pressure.[10-12] Finally, functional and structural responses of resistance vessels are coupled. Thus, the group of Bakker and Van Bavel has shown that long-lasting tone is a drive for inward remodeling of resistance vessels in a wide range of in vitro and in vivo settings.[13] In a systems analysis of structural and functional control of resistance vessels, Guvenc Tuna and Van Bavel demonstrate the key importance of smooth muscle cells contraction in the final regulation of vascular caliber, wall thickness, and organization of the matrix and smooth muscle cells in the wall.[14]

Contraction of vascular smooth muscle cells is an active process wherein the myosin light chains must be phosphorylated before shortening can occur.[11,12] The contraction is triggered by a rise in cytosolic Ca^{2+} and initiated by phosphorylation of the serine 19 residue of the myosin regulatory light chain by a specific Ca^{2+} calmodulin–myosin regulatory light chain kinase complex.[11,12] 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.[11,12] The functional tonus of the vessel is thought to be influenced by among others nervous and humoral factors, but it is in general not detailed which intracellular events lead to the development of the increased tone in hypertensive resistance arteries. Possibly, inherent differences in the strength and endurance of contraction affect the level of tone independent from external stimuli.

For the last 20 years we and others have intensely investigated one of the key factors of intracellular energy supply, the creatine kinase system, and its role in hypertensive disorders.[15-24] In this thesis I will focus on the role of creatine kinase in resistance artery contractility and its possible therapeutic implications.

II. The creatine kinase system

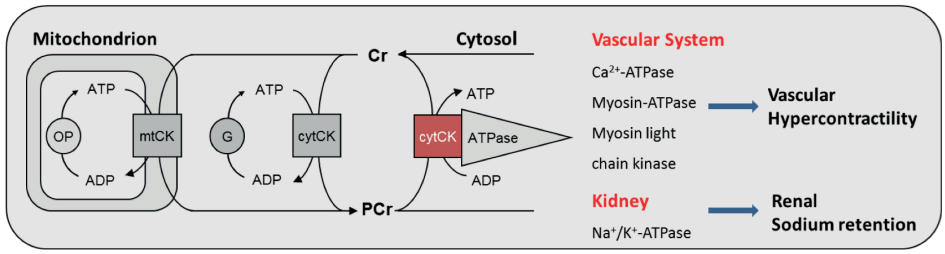
The enzyme creatine kinase (CK, EC 2.7.3.2) regenerates and distributes ATP to subcellular locations of energy demands by catalysing the rapid and reversible transfer of a phosphate group from creatine phosphate to ADP, thereby forming creatine and ATP:[15-20,25-29]

$$\text{Creatine phosphate} + \text{MgADP} \leftrightarrow \text{Creatine} + \text{MgATP}$$

Creatine kinase is found in different tissues, located in the cytosol and mitochondrion of cells.[25-29] The cytosol contains the CK-B (brain type) and CK-M (muscle type) isoforms, while the mitochondrial creatine kinase (mtCK) isoforms consist of the ubiquitous mtCK in non-muscle tissues and sarcomeric mtCK in striated muscle.[25-29] Combining the two cytosolic isoforms give the three typical dimeric MM-, MB- and BB-CK isoenzymes.[25-29] The MM-CK isoenzyme is specific for differentiated sarcomeric muscle, BB-CK is found in brain and in a variety of other tissues and MB-CK is expressed in the heart.[25-29]

The CK isoenzymes are associated with sites of ATP production and ATP consumption.[25-29] ATP is synthesized in the mitochondria and is transported by mtCK in the mitochondrial intermembrane space to creatine (Cr) to yield ADP plus phosphocreatine (PCr).[25-29]
PCr is shuttled through the mitochondria and diffuses through the cytosol to the sites of ATP consumption.[25-29] Cytosolic CK accepts the PCr and regenerates ATP, which can be used by the ATPases (Figure 1).[25-29] Thus, CK is located at subcellular energy producing and consuming compartments, such as in the mitochondrion and near glycolytic enzymes, as well as near proteins involved in force generation at (acto)myosin-ATPase, myosin light chain kinase and other ATPases such as Na^+/K^+-ATPase and Ca^{2+}-ATPase (Figure 1).[15,25-29]

Figure 1. The creatine kinase system



Legend: Cellular mechanism of the creatine kinase system modified after schlattnet et al.[25] Cytosolic creatine kinase synthesizes and transports phosphocreatine to ATPases including Na^+/K^+-, Ca^{2+}-, and myosin ATPase, where it rapidly regenerates ATP in situ, maintaining a high ATP to ADP ratio near the ATPase. Mitochondrial creatine kinase synthesizes phosphocreatine from mitochondrial ATP creating a phosphoryl group shuttle towards the cytoplasm. Cr, creatine; PCr, phosphocreatine; CKcyt and CKmit, cytoplasmic and mitochondrial CK; G, glycolysis; OP, oxidative phosphorylation

III. The role of creatine kinase in resistance artery contractility

There is increasing evidence that the enzyme CK, the central regulatory enzyme of energy metabolism, is intimately involved in the regulation of blood pressure.[15-24]
The CK-system is of particular importance in tissues that display high and variable rates of ATP turnover, including skeletal muscle, the cardiovascular system, brain, and the kidney.[25-29] ATP regenerated by CK is preferentially used to fuel highly energy-demanding processes such as cardiovascular contractility and increase renal tubular ability to retain salt, thus promoting high blood pressure levels (Figure 1).[15-29]

Creatine kinase is specifically located at subcellular energy producing compartments, including the mitochondrion and near glycolytic enzymes.[25-29] Due to this specific localization, ATP generated by glycolysis and oxidative phosphorylation, is shuttled as phosphocreatine to subcellular locations of ATP utilization, where ATP is regenerated (Figure 1).[25-29] Evidence indicates that CK enhances vascular contractility and increases blood pressure through rapid regeneration of ATP near cytosolic ATP-utilizing enzymes such as Na^+/K^+-ATPase Ca^{2+}-ATPase, and myosin ATPase involved in pressor responses.[25-29] Vascular CK acts as an energy transducer at the smooth muscle contractile proteins, supplying ATP for the contractile process.[25-29]

There is evidence that hypertension in individuals with high plasma CK activity is more severe and more resistant to treatment.[30,31] Importantly, CK is a major predictor of failure of hypertension treatment in the general population.[30,31] In accordance with this, serum CK was found to be a main predictor of blood pressure in the general population, independent of age, sex, BMI, and ethnicity.[16,18] The crude increase in systolic and diastolic blood pressure in the general population is 14 and 8 mm Hg per tenfold increase in creatine kinase plasma activity.[16] This estimate was replicated in other settings.[18,20] Enhanced resistance artery contractility due high smooth muscle creatine kinase activity was proposed to contribute to the higher blood pressure levels.[15-24] However, hitherto, there are no data to substantiate this hypothesis. Therefore, in this thesis I will study

the role of creatine kinase in resistance artery contractility and explore therapeutic implications.

IV. Outline of this thesis

This thesis consists of two parts. In **part I** we focus on the role of CK in resistance artery contractility. Evidence that the level of vascular CK is associated with contractility responses and blood pressure is lacking. First, we need data to establish whether high vascular CK is associated with blood pressure. In **Chapter 2** we systematically review the evidence on the association between plasma CK activity and blood pressure categories (normotension and hypertension, subdivided in treated controlled, treated uncontrolled and untreated hypertension). In **Chapter 3** we assess whether expression of the creatine kinase B gene in resistance arteries is associated with blood pressure. In **Chapter 4** we assess whether microvascular contractility across the spectrum of normotension and hypertension in resistance arteries of humans is CK dependent.

In **part II**, we investigate whether modulation of the CK system might become a target for blood pressure lowering in humans. In **Chapter 5** we assess whether inhibiting the creatine kinase system with a specific blocker reduces blood pressure in the spontaneously hypertensive rat. **Chapter 6** describes a protocol of a randomized placebo controlled trial with a low dose of the specific CK inhibitor in healthy man and in **Chapter 7** we report the results of this first-in-human trial. **Chapter 8** provides a summary and general discussion of the thesis.

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Chapter 2

Creatine kinase and blood pressure: a systematic review

Lizzy M. Brewster, Fares A. Karamat, Gert A. van Monfrans.
Medical Sciences, accepted for publication on 26 March 2019

Abstract

Background: Hypertension is the main risk factor for premature death. Although blood pressure is a complex trait, we have showed that the activity of the ATP-generating enzyme creatine kinase (CK) is a significant predictor of blood pressure and of failure of antihypertensive drug therapy in the general population. In this report, we systematically review the evidence on the association between this new risk factor CK and blood pressure outcomes.

Method: We used a narrative synthesis approach and conducted a systematic search to include studies on non-pregnant adult humans that address the association between plasma CK and blood pressure outcomes. We searched electronic databases and performed hand search without language restriction. We extracted data *in duplo*. The main outcome was the association between CK and blood pressure as continuous measures. Other outcomes included the association between CK and blood pressure categories (normotension and hypertension, subdivided in treated controlled, treated uncontrolled and untreated hypertension).

Results: We retrieved 139 reports and included 11 papers from 10 studies assessing CK in 34,578 participants, men and women, of African, Asian, and European ancestry, aged 18 to 87 y. In 9 reports, CK was associated with blood pressure levels, hypertension (vs normotension), and/or treatment failure. The adjusted increase in systolic blood pressure (mmHg/log CK increase) was reported between 3.3 [1.4 to 5.2] and 8.0 [3.3 to 12.7]; and the odds ratio of hypertension with high vs low CK ranged between 1.2 and 3.9. In addition, CK was a strong predictor of treatment failure in the general population, with an adjusted odds ratio of 3.7 [1.2 to 10.9].

Discussion: This systematic review largely confirms earlier reports that CK is associated with blood pressure and failure of antihypertensive therapy. Further work is needed to address whether this new risk factor is useful in clinical medicine.

Keywords: (MeSH Unique ID): creatine kinase; Registry Number: EC 2.7.3.2 (D003402); blood pressure (D001794); hypertension (D006973); systematic review (D000078182); continental population groups (D044469); antihypertensive drugs (D000959)

Introduction

Hypertension is the main risk factor for cardiovascular disease, chronic kidney disease and premature death worldwide.1 Global prevalence of raised blood pressure in adults is estimated to be 35 to 40%.1 The number of adults with raised blood pressure increased from 594 million in 1975 to 1.13 billion in 2015, with the increase largely in low-income and middle-income countries, rendering hypertension an important global public health challenge.[1,2]

Raised blood pressure is estimated to cause 7.5 million deaths, about 13% of the total of all deaths and around 60% of cardiovascular mortality, accounting for 57 million disability adjusted life years (DALYS) or around 4% of total DALYS. Blood pressure levels have been shown to be positively and continuously related to the risk for stroke and heart disease with risk starting as low as 115/75 mmHg systolic/diastolic blood pressure. Complications of raised blood pressure further include heart failure, peripheral vascular disease, renal impairment, retinal hemorrhage and visual impairment, which could largely be prevented by reducing blood pressure to 139/89 systolic/diastolic blood pressure or lower.[1,3]

Blood pressure is a complex trait, affected by environmental, psychological, as well as biological factors, including education and income levels, psychosocial stress, genes, fetal and early childhood nutrition and growth, and nutrition and body mass in later life.[4] In 2000, a new genetic factor for hypertension and cardiovascular disease was proposed by our group, the activity of the ATP-generating enzyme creatine kinase (CK, EC 2.7.3.2).5 The enzyme transfers a phosphoryl group from creatine phosphate to ADP, thereby forming creatine and ATP, catalysing the reaction:[5]

$$\text{Creatine phosphate} + \text{MgADP} \leftrightarrow \text{Creatine} + \text{MgATP}$$

We proposed that high CK promotes hypertension through enhanced vascular contractility and salt retention in the kidney (Figure 1). Subsequently, CK was shown to be a significant independent predictor of blood pressure levels and failure of antihypertensive therapy in a random sample of a multi-ethnic population of Amsterdam, the Netherlands. The subjects with the highest CK activity levels were men, persons with obesity, and persons of West-African ancestry.[6,7] Crude blood pressure increase per log CK increase was substantial at 14 mm Hg for SBP and 9 mm Hg for DBP.6 Since then, several other studies have reported data on this association. In this report, we systematically review the evidence on the association between this new risk factor CK and blood pressure.

Methods

We systematically reviewed the evidence on the association between CK and blood pressure in humans with standardized methods designed to conduct reviews of etiology.[8] Because of the expected heterogeneity in outcomes, we used a "narrative synthesis approach", where a narrative summary of the findings of studies is used to perform the

data synthesis, with safeguards in place to avoid bias resulting from the undue emphasis on one study relative to another.[9]

Figure 1. Creatine kinase and pressor responses.

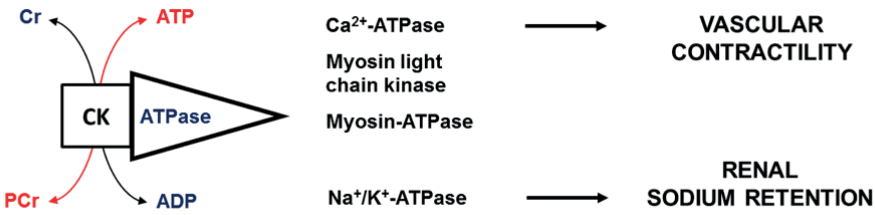

Figure Legends: This figure depicts the proposed pathophysiology of high blood pressure with high creatine kinase (CK). CK is tightly bound near ATPases such as calcium, sodium/potassium and myosin ATPase, where the enzyme rapidy buffers the ADP generated by these ATPases into ATP, utilizing phosphocreatine. Thus, greater CK activity near these ATPases is thought to promote vascular contractility and ability to retain salt.[5,6] This places the individual with high CK at greater risk to develop hypertension, with greater resistance against blood pressure-lowering therapy.[6,7]

We used a framework with 4 elements to characterize the narrative synthesis based on Rodgers et al,[9] a). Present the theory on the pathophysiology of the association; b). Conduct a preliminary synthesis; c). Explore relationships within and between studies; and d). Critically assess the robustness of the synthesis product. Finally, we discuss the relevance of the aggregated evidence for the design of further etiological and clinical studies.[8]

Based on the protocol presented here, we sought to identify through systematic searching, all studies which provided original data in non-pregnant adult humans on the association between tissue or plasma CK and systemic blood pressure in population (sub) groups. We excluded case reports. We critically appraised the retrieved studies in duplo, and synthesized the evidence found, including the following aspects: population, exposure (CK), and outcome (blood pressure-related outcomes).[8] We also analyzed confounding variables or moderators that may impact on the results.[8,9] Depending on the available data, the results were planned to be synthesized in a combination of narrative and tabular summaries.[8]

Systematic searches were conducted in February 2019, in electronic databases (Embase, PubMed, Literatura Latino-Americana y del Caribe en Ciencias de la Salud (LILACS), African Index Medicus, and IndMED) from their inception through February 2019. Databases differed in technical search options, but a typical search strategy was, "(Creatine kinase OR Creatine phosphokinase OR HyperCKemia) AND (hypertension OR blood pressure OR Cardiovascular). We intended to include only studies providing original data on the association between CK and blood pressure measures, and not studies merely reporting both parameters. Therefore, in developing this search strategy, we searched on title words where technically possible for a more relevant search yield, since a large majority

of papers on CK and heart disease are on myocardial infarction.

Search yields from the different databases were considered and analyzed separately to prevent merging errors and to enhance the retrieval of reports. We took further care in preventing bias in retrieval and inclusion of studies, by including reports without language restriction and in performing hand search. Data were extracted independently by two authors (LB and FK). Where relevant, European, African, Asian or other descent (ancestry, or ethnicity) were defined as respectively of European, sub-Saharan African or Asian heritage as indicated by the authors of the eligible papers.

The main outcome was the association between CK as a continuous measure and blood pressure as a continuous measure, as reported by the authors. Other outcomes included the association between CK and blood pressure categories (normotension and hypertension as defined by the authors, subdivided in treated controlled, treated uncontrolled and untreated hypertension).

We also assessed important determinants of plasma CK activity, such as physical exercise and the method of CK assessment. Plasma CK increases after exercise, in particular during the first 3 days, and this may dilute the association of CK with blood pressure.[6] Therefore, we collected data on whether resting CK was estimated. Furthermore, as CK estimation is a bioassay, we also collected information on whether the method of CK estimation as reported in the papers (or from the manufacturer of the device described in the paper) was according to the standardized methods of the International Federation of Clinical Chemistry, IFCC as previously described.[10]

Predefined subgroups were based on sex, ancestry group, and geographical location. We expected heterogeneity in the participants' characteristics, CK comparisons, and outcomes, and therefore planned to describe the findings as reported by the authors. Data in square brackets are 95% confidence intervals, and in parentheses are standard errors, unless indicated otherwise.

Results

Systematic search yield

The study flow is depicted in Figure 2, and the included studies are in Table 1.[6,7,11-19] We retrieved 135 reports from electronic databases and included 7 reports from 6 studies.[6,7,11,15-17,19] We additionally retrieved 4 papers through handsearch,[12-14,18] 3 of which were not included in electronic databases.[12,13,18] In total, we included 11 papers from 10 studies.[6,7,11-19]

Table of Included Studies (Table 1).

Author, year	Population	Ancestry	Country	N	Age*	CK estimations			Outcome	Effect size¶
						Resting†	Device	IFCC		
Blood Pressure										
Brewster 2006[6]	Random population sample	African Asian European	Netherlands	1444	35-60	Yes	Roche-Hitachi Systems	Yes‖	CK associated with SBP and DBP	**CK T1 (<88) vs CK T3 (>/=145)** SBP 122.5 (1.0) vs 130.6 (0.9) DBP 79.2 (0.6) vs 84.8 (0.6) **Univariable** SBP: + 13.9 [9.6 to 18.3]/log CK DBP: + 9.3 [6.8 to 11.9]/log CK **Multivariable** SBP + 8.0 [3.3 to 12.7]/log CK DBP + 4.7 [1.9 to 7.0]/log CK
Johnsen 2011[11]	Population sample	European	Norway	12,776	30-87	No‡	Modular P, Roche	Yes	CK associated with SBP and DBP	**CK T1 vs CK T3** SBP 134.4 (0.4) vs 138.2 (0.4) DBP 76.3 (0.2) vs 79.8 (0.2) **Multivariable** SBP + 3.3 [1.4 to 5.2]/log CK DBP + 1.3 [0.3 to 2.3]/log CK
Mels 2016[15]	Teachers	African	South Africa	405	45 (0.5)	No	Beckman UniCel DxC800; Konelab 20i	Yes	Only subgroup analysis	CK only associated with BP in women of European ancestry. Adjusted R2 = 0.46; β = 0.17; p = 0.03
Yen 2017[17]	Population health survey	Asian	Taiwan	4562	49 (0.2)	Yes	Modular P, Roche	Yes	CK associated with SBP and DBP	**CK Q1 (<69) vs CK Q4 (>/=128)** SBP 118.6 (0.3) vs 124.2 (0.3) DBP 73.1 (0.2) vs 76.6 (0.2) **Univariable** SBP + 6.5 [5.2 to 7.7] CK/10 mmHg DBP + 10.1 [8.0 to 12.1] CK/10 mmHg **Multivariable** SBP + 1.68 CK/10 mm Hg

Hypertension										
Brewster 2008[19]	Cases with hyperCKemia vs population controls	European	Netherlands	46 (controls 22,612)	18-67	Yes	Modular P, Roche	Yes ‖	High CK associated with hypertension	**Odds ratio of hypertension§** Crude: 3.9 [2.2 to 6.9] Adjusted: 2.0 [1.1 to 3.8]
Johnsen 2011[11]	Population sample	European	Norway	12776	30-87	No‡	Modular P, Roche	Yes	CK higher with HT	CK higher in persons using anti-HT drugs vs none (104 vs 99)
Brewster 2013[7]	Random population sample	African Asian European	Netherlands	1444	35-60	Yes	Roche-Hitachi Systems	Yes ‖	CK higher in HT vs NT	**Odds ratio of hypertension** CK T1 (<88) vs CK T3 (>/=145) HT prevalence: 26.8 vs 41.2% Odds ratio 1.9 [1.5 to 2.5] **CK in HT vs controls** CK 145.9 (7.0) HT vs 126.8 (2.5) controls
George 2016[14]	Population study	African Asian European	USA	10,096	>20	No	Beckman UniCel DxC800	Yes	Only subgroup analysis	**Odds ratio of HT (CK dichotomized, ULN)**** Men: 1.2 [0.8 to 1.7] Women: 1.4 [1.0 to 2.1]
Yen 2017[17]	Population health survey	Asian	Taiwan	4562	49 (0.2)	Yes	Modular P, Roche	Yes	CK higher in HT vs NT	**CK in HT vs controls** CK +20.7 [15.8 to 25.6] in HT vs controls
Sukul 2018[18]	Hypertensives vs controls	Asian	India	115	25-60	Yes	Roche diagnostics	Yes ‖	CK higher in HT vs NT	**CK in HT vs controls** CK 199.6 (16.4) HT vs 72.7 (4.0) controls
Kumar 2013[12]	Hypertensives vs controls	Asian	India	150	40-90	No	NR	NR	CK MB higher in HT vs NT	**CK MB in HT vs controls** 21.5 (4.0) HT vs 17.2 (2.4) controls
Emokpae 2017[16]	Hypertensives vs controls	African	Nigeria	340	28-62	No	Selectra Pro S	Yes	CK MB higher in HT vs NT	**CK MB in HT vs controls** 51.6 (3.0) HT vs 15.0 (0.8) controls

Treatment Failure										
Johnsen 2011[11]	Population sample	European	Norway	12776	30-87	No‡	Modular P, Roche	Yes	CK not significantly higher in uncontrolled vs controlled HT	**CK in controlled vs uncontrolled HT** 101 vs 110††
Brewster 2013[7]	Random population sample	African Asian European	Netherlands	1444	35-60	Yes	Roche-Hitachi Systems	Yes ‖	CK higher in uncontrolled vs controlled HT	**CK in controlled vs uncontrolled HT** 124.3 (10.9) vs 157.9 (9.4) **Odds ratio of treatment failure** CK T1 (<88) vs CK T3 (>/=145) HT treatment failure 46.7% vs 72.9% Odds ratio 1.6 [1.3 to 1.9] **Adjusted odds ratio treatment failure** 3.7 [1.2 to 10.9]/log CK
Luman 2015[13]	Hypertensives	Asian	Indonesia	82	>18	No	Roche-Hitachi cobas analyzer	Yes	CK higher in uncontrolled vs controlled HT	**Mean CK in controlled vs uncontrolled HT** 81.8 (3.3) vs 132.2 (6.2) **High CK (T3 CK>109.33 U/L)** Controlled hypertension 18.5% Uncontrolled hypertension 81.5%
Sukul 2018[18]	Hypertensives vs controls	Asian	India	115	25-60	Yes	Roche diagnostics	Yes ‖	CK higher in uncontrolled vs controlled HT	**CK in controlled vs uncontrolled HT** 99.6 (4.5) vs 313.9 (22.5)

Legend. Studies reporting creatine kinase (CK) and blood-pressure outcomes. Blood pressure is in mm Hg, and CK in (I)U/L. Where applicable, data are rounded to one decimal place. Data in square brackets are 95% confidence intervals, in parentheses are standard errors, and outcomes are significant at P<.05, unless stated otherwise. *Age in years, †Test under resting conditions, as defined by the authors. ‡Outcomes adjusted for habitual exercise. IFCC, CK estimated according to the International Federation of Clinical Chemistry guidelines,[20] reported by 3 studies; ‖ [6,7,18,19] we retrieved online information for other studies. NR, not reported. SBP, DBP, systolic, diastolic blood pressure; HT, hypertension (as defined by the author; generally blood pressure >139 systolic or 89 diastolic, or the use of antihypertensive drugs). NT, normotension. CKMB, CKMB isoenzyme; ¶Multivariable analyses as reported, mostly including sex, age, BMI, among other variables; T1, T3 low vs high CK tertile; Q1, Q4 lowest vs highest CK quartile; §Compared to population controls. **ULN, upper limit of normal (334 in men, 199 in women).[14] ††No SE reported, p=0.1, direction (one or two-sided) not reported.

Figure 2. Paperflow below

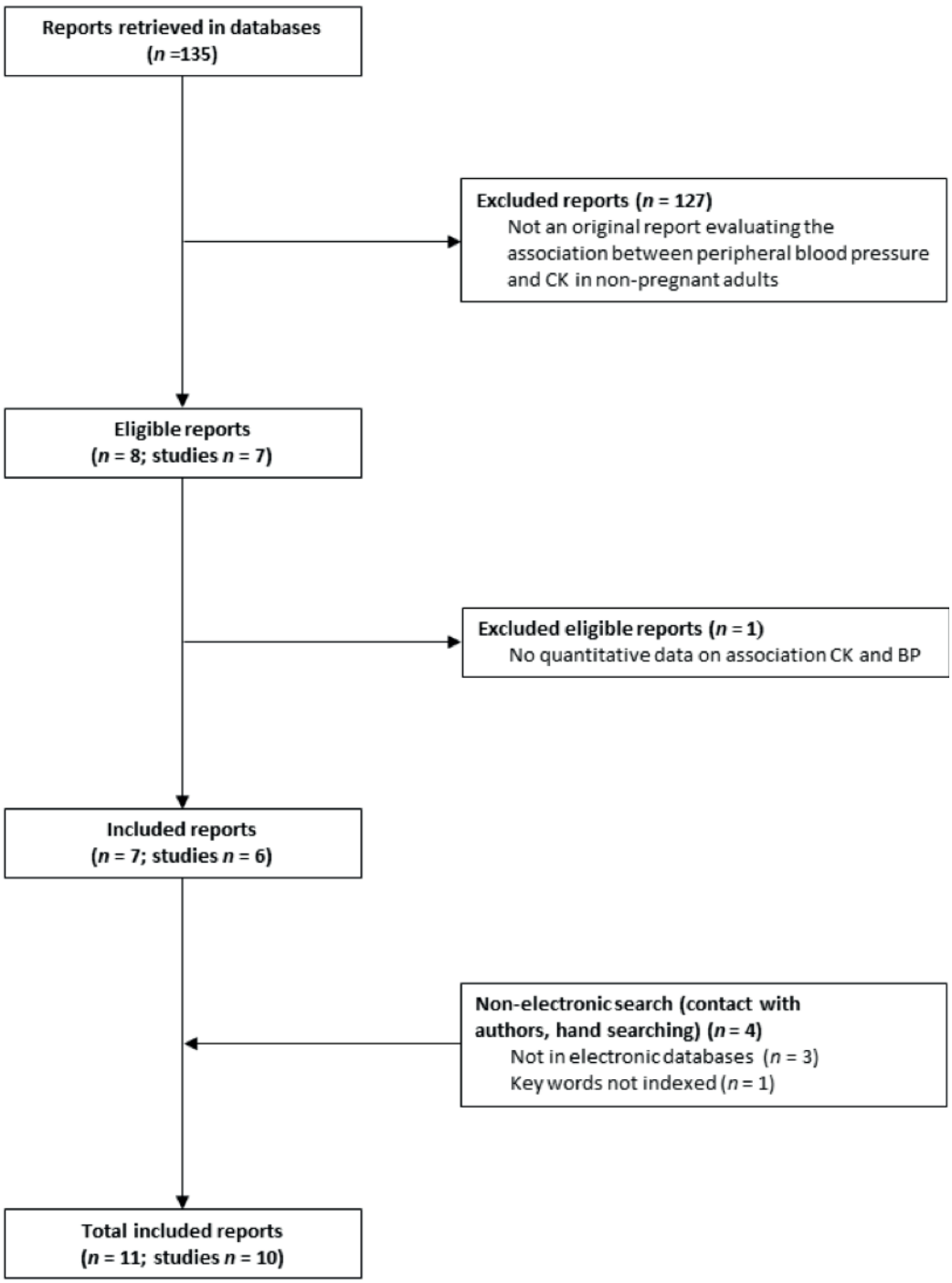

Figure Legends: The Figure depicts the number of retrieved, eligible, and included reports and the yield of the hand search. The 11 included papers are reports from 10 studies.

Studies and Participants

The studies assessed CK in 34,578 participants, men and women of African, Asian, and European ancestry aged between 18 and 87 y. The included studies were population studies with relatively large sample sizes (n=4),[6,7,11,14,17] or clinical studies (n=5) among hypertensives and controls.[12,13,16,18,19] One smaller study included participants by profession.[15]

Exposure

CK was estimated under resting conditions (to control for exercise-induced hyperCKemia) in 4 out of 10 studies (2 population studies).[6,7,17-19] Two additional population studies did not restrict exercise, but collected information on either the participants habitual exercise levels,[11] or exercise in the 3 days before the CK test.[14] Definition of exercise varied, in "exercise or work in the past 3 days that causes large increases in breathing or heart rate if they are done for at least 10 minutes continuously,"[15] "hard leisure physical exercise (sweating or out of breath) at least 2 h/week,"[11] or "participants were instructed to abstain from heavy exercise during 3 days before the test. Walking, driving, and normal daily activities were allowed."[10] In one study subjects were asked to avoid vigorous exercise or intramuscular injection 48 hours prior to study enrollment.[17]

CK estimation was performed with commercially available analyzers for medium- to high-volume laboratories in all but one study that did not report the method used to estimate CK.[12] Most studies (6 out of 10) used a Roche® device (Table 1).[6,7,11,13,17-19] Only three studies reported the application of IFCC guidelines.[6,7,10,18,19] We retrieved additional information from the supplier companies in the 7 other studies, and all claimed traceability to the IFCC reference method[20], provided the manufacturer's reagents are used (Table 1).

Comparisons and Outcomes

Studies reported associations of CK with blood pressure as continuous measures, categorized blood pressure outcomes including normotensives vs hypertensives, or the association between CK and failure of antihypertensive treatment (Table 1). The data can be observed to be heterogeneous in participants, CK estimations, and comparisons, as expected. Therefore, we describe the data in a narrative synthesis as planned.[9]

In 9 out of 11 papers, the direction of the outcome indicated that CK or CK MB are positively associated with blood pressure levels, hypertension (vs normotension), or treatment failure (vs controlled hypertension) (Table 1). Although most studies did not provide a sample size calculation, the magnitude and distribution of the outcome in relation to the sample size were sufficient to be statistically significant in the populations studied, except for 2 studies, George et al. and Mels et al. which report only subgroups under non-resting conditions (Table 1).[14,15]

Unlike the other included papers, the methodology of the report by George et al. was

not designed to address the association of CK with blood pressure. The authors associate hypertension by sex with dichotomized CK levels at the cutoff point provided by the manufacturer, at 334 for men and 199 for women. This is remarkable, as the authors report in the same paper that these upper limits of normal (ULN) levels are incorrect. The authors report ULN for CK (IU/L) of 1001 for black men, 382 for white men, 487 for black women, and 295 for white women. The authors do not explain why they chose to dichotomize CK at 334 for men and 199 for women.

The study of Mels et al.[15] also addressed only subgroups, by sex and ancestry. Mean CK (95th percentile) was respectively 127.0 (427), 115.0 (245), 75.9 (195), 62.8 (123) IU/L for African ancestry men, European men, African ancestry women, and European women. However, at this relatively small sample size (around 100 in each group), difference in CK by ancestry did not reach statistical significance in men, and CK was only associated with blood pressure in white women (Table 1).

Notably, George and Mels et al. were the only studies that used Beckman UniCel DxC800 (Beckman and Coulter, Germany) to estimate CK. Mels et al. additionally used the Konelab 20I Sequential Multiple Analyzer Computer (Thermo Scientific, Vantaa, Finland). Both devices have been associated with suboptimal performance, in particular in CK estimations,[21-24] and together with the non-resting conditions this might have contributed to the reported outcome of these 2 studies, which differs in magnitude from the majority of studies included.

Subgroup analysis

The association between blood pressure and CK was reported in European,[6,7,11,19] Indonesian,[13] Taiwanese,[17] Indian,[6,7,12,18] and West-African[6,7,16] populations. CK is higher in men, persons with overweight and persons of West-African-ancestry, but the studies reporting an association between CK and blood pressure outcomes provided evidence that such association is independent of sex, BMI, and ancestry, where applicable.[6,7,11,17,19]

Discussion

In this systematic review, we confirm the hypothesis,5 and subsequent finding6 of an association between CK and blood pressure. The data from 10 studies, presented in 11 papers were heterogeneous in characteristics of the participants, clinical conditions surrounding the estimation of plasma CK, and primary outcomes, but 9 out of 11 papers report that CK as a continuous measure is associated with blood pressure, the presence of hypertension, and/or failure of antihypertensive therapy.[6,7,11-13,16-19] In one study, the point estimate indicated an association between dichotomized CK and hypertension by sex, but the outcome did not reach statistical significance.[14] Another study found the association only in white women.[15] Both studies had used the Beckman UniCel DxC800 to estimate enzyme activity, which has shown less favorable results in quality assessments than the Roche device which was used in most included studies. This might have impacted the magnitude and linearity of the CK estimation in these studies.[20-24]

Normal tissue releases CK proportionate to the intracellular CK concentration, a physiologic process that occurs without tissue damage, as summarized by Brewster et al.[6] Therefore, plasma CK in healthy persons at rest reflects tissue CK.[6,7,25] However, with exercise, lymphatic flow increases and CK from the interstitial space may enter the circulation rather abruptly, where it is cleared by the liver in around 3 days.[6] With frank tissue damage, such as after eccentric exercise, where the muscle contracts and stretches at the same time, or with myocardial infarction or brain trauma, large quantities of CK enter the circulation, proportional to intracellular CK and the damaged area.[6,26,27] Our group recently showed that these large increases in plasma CK might induce perturbations in coagulation, as circulating CK will reduce ADP needed for platelet aggregation.[26,27] Hence, such high CK levels might induce coagulopathy and bleeding risk.[26,27]

High tissue CK is thought to lead to a phenotype with greater vascular contractility and enhanced salt retention, through greater ATP buffer capacity at ATPases involved in ion transport and contractile responses.[5,6,27-30] CK is tightly bound near these ATPases, including Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase, and myosin ATPase, where it rapidly provides ATP in situ. In skeletal muscle, high CK Type II muscle fibers display reduced cytoplasmic uptake of glucose and fat, which is a risk factor for glucose intolerance, insulin resistance and obesity.[31]

Thus, based on the existing evidence, the high CK phenotype might carry greater risk for hypertension, as well as bleeding risk and obesity.[27] None of the included studies evaluated bleeding risk. A few published studies report an association between CK and obesity,[14,31-33] but this was not the topic of our review. CK isoenzyme distribution was reported to be normal in hypertensives,[19] as expected in the absence of tissue damage.

Strengths and limitations of this study

The main strength of this narrative review is that it summarizes and discusses the existing evidence on the biologically plausible association of CK with blood pressure, indicating that after the initial report in 2006,[6] this has been found in different populations across the world.

We use rigid systematic review methodology[8,9] and took great care to retrieve and include all relevant studies, including studies not in PUBMED, studies not indexed in electronic science databases, and studies that did not find the association, without using any language restriction. We did not find evidence of bias towards a preferred outcome in the papers, the risk which is lower with observational, non-intervention studies, where the researcher only observes certain characteristics of the sample population and records the data.[8] However, it is well known that scientific papers with “negative” findings are less likely to be published.[8,9] In particular with narrative syntheses, one needs to critically assess the presence of confounding variables or moderators that may impact on the results, and we cannot exclude that publication bias affected the search yield of this review.[8,9]

A further limitation of the included studies is that we are not well informed regarding the comparability and quality of the CK assays.[20-24] Results of blood samples assayed by routine measurement procedures should represent the true value of the sample. However, CK is not measured in moles or grams, but as catalytic, functional performance in a bioassay. The results represent a relative measure of (re-) activated enzyme activity, a method standardized by the IFCC.[20] Importantly, the level of reactivated activity and linearity of the estimations across the spectrum of low, mid, and high CK activity may vary. This may lead to overestimation, underestimation, or distortion of linearity of the results at different levels of enzyme activity, across, but also within devices and laboratories. Therefore, laboratories need to regularly assess the quality of their CK estimation. With quality assessment, the quality of the test at low through high concentrations of standard reagents is addressed. Devices may also differ in their performance during independent quality assessments. The Roche® devices scored well in quality tests.[20-24] Most of devices used in the include studies were from Roche®, but two studies used a Beckman UniCel DxC800 device, of which the performance was reported suboptimal in independent quality assessments.[21-23] This might have affected the representation of the spectrum of CK values into the test results, especially the linearity at the extremes of the CK spectrum.[20-24] We are not well informed whether the range measured in the included studies was comparable across measuring devices, and whether linearity for all assays was acceptable and comparable over the range tested.[21-24] Still, the association found between CK and blood pressure is robust and was reported across most devices used.

Furthermore, only 4 studies standardized CK assessments to resting conditions.[6,7,17-19] With heavy, exercise, plasma CK does not well reflect tissue CK, and the association with blood pressure can be expected to be attenuated under these conditions.[6,30] All studies reporting resting CK found an association with blood pressure outcomes.[6,7,17-19] In addition, CKMB in plasma is less dependent on exercise levels than total CK in plasma, and both studies assessing the cardiac isoenzyme CKMB in plasma, showed an association of CK with blood pressure outcomes.[12,16] Thus, although the molecular mechanisms of the association between CK and blood pressure outcomes are well described,[5-7,27] evidence indicates that the quality of the CK estimation is relevant when analyzing the association, in taking care to test under resting conditions when using plasma CK as a surrogate for tissue CK, and in using standardized IFCC methods. The field of CK research and cardiovascular disease would benefit from the further development of non-invasive assessments of tissue CK for clinical use, such as ^{31}P-magnetic resonance spectroscopy[34] of the calf muscles.

In summary, the majority of studies included in this systematic review confirm the association between CK and blood pressure. The association is based on evidence that ATP-buffer capacity is relevant for the generation of blood pressure. Relatively high CK activity may carry risk for hypertension that is difficult to treat, and reported links of CK with obesity and bleeding risk are also biologically plausible. However, the lack of information regarding the comparability and quality of the CK essays used in the included studies is a limitation of this review. Further studies will need to address the usefulness for clinical medicine of this new and emerging field of CK related-hypertension and cardiovascular disease, which has recently been substantiated by experimental evidence

providing evidence of a causal relationship,[27,28,30,35] including reduction of blood pressure with CK inhibitors.[35]

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Author Contributions: Conceptualization FK and LB; Methodology, FK, LB and GvM; Systematic search, study retrieval and data extraction FK and LB, Data synthesis FK, LB and GvM; Writing – Original Draft Preparation, LB; Writing – Review & Editing, FK, LB and GvM; Visualization, FK and LB. All authors have seen the final manuscript and agreed to its content and submission of the paper.

Conflicts of Interest: LMB is an inventor on NL patent WO/2012/138226 (filed).

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Chapter 3

Resistance artery creatine kinase mRNA and blood pressure in humans

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Hypertension. 2014; 63:68-73

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 due to high resistance artery creatine kinase-BB rapidly regenerating ATP for vascular contractility. Therefore, we assessed whether creatine kinase isoenzyme 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 y (SE 1.6); mean systolic/diastolic blood pressure, 144.8 (8.0)/86.5 (4.3) mm Hg. Arteriolar creatine kinase isoenzyme mRNA was assessed using quantitative real-time PCR.

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% CI, 0.6 to 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 to normotensives; normalized copy numbers respectively 19.3 (SE 2.0) vs 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.

Key words: creatine kinase, hypertension, microcirculation, resistance artery, genetics (human)
247 words

Introduction

Hypertension is an important worldwide public-health challenge.[1-3] It is a common disease, affecting over 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.[1-3] The pathogenesis of hypertension is multifactorial, and environmental and biological circumstances contribute to the occurrence of the disease.[1-4] We proposed that creatine kinase (CK), the central regulatory enzyme of energy metabolism, is the final common pathway leading to pressor responses.[5,6] The enzyme regenerates and distributes ATP to subcellular locations of energy demands, catalyzing the reaction:

$$MgADP + Pcreatine + H^{+} \leftrightarrow MgATP + creatine.^{[5-11]}$$

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.[5-10] In accord with this, serum CK was found to be the main predictor of blood pressure in the general population.[6] This was proposed to be due to high tissue CK, primarily the resistance artery CK-BB isoenzyme rapidly regenerating ATP for vascular contractility.[6] 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. Sitting blood pressure was measured at the outpatient clinic with the Datascope 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), respectively encoded by the CKB gene on human chromosome 14q32 and the CKM gene on 19q13.32. The enzymatic functional form can be either a homodimer (BB or MM)

or a MB heterodimer, thus creating 3 cytosolic isoenzymes.[5,9,10,12] CK is also present in the mitochondrion 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[5,12] 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 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 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, $NaHCO_3$ 24.8, KCl 4.6, KH_2PO_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 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 CK. The transcription of CK genes was normalized to the reference gene 26S proteasome non-ATPase regulatory subunit 4 (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.

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

Table 1. Primers used in the quantitative real-time polymerase chain reaction

Transcript	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	UPL
CKB	TTCTCAGAGGTGGAGCTGGT	AGGCATGAGGTCGTCGAT	77
CKM	CCCACAACAAGTTCAAGCTG	GGCCATGTGGTTGTTATGTTT	63
CKMT1	GGTAACATGAAGAGAGTGTTTGAAAG	CAGCCACGTTCTTGGATAAGT	39
CKMT2	TGAACCGGCAGAAAGTGTG	CGCAGGTCTGGGTAGTCTG	32
PSMD4	GGCAAGATCACCTTCTGCA	CTTCCCACAAAGGCAATGAT	21

Legend CKB indicates cytoplasmic brain-type creatine kinase; CKM, cytoplasmic muscle-type creatine kinase CKMT1 and CKMT2 are respectively ubiquitous and sarcomeric mitochondrial creatine kinase. PSMD4, 26S proteasome non-ATPase regulatory subunit 4; and UPL, indicates the number of the Universal ProbeLibrary probe (Roche).

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. 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. As parametric analysis may not be accurate with small sample sizes, and non-parametric analysis may lack power to detect a significant difference, we prespecified to use parametric statistics as our primary analysis (i.e. arithmetic mean with standard error (SE); Pearson product-moment correlation coefficient (r), the unpaired t test, and 1-way ANOVA with the Bonferroni procedure as a post-hoc analysis); and to reanalysed the data as a sensitivity analysis with non-parametric methods (i.e. median with interquartile range; Spearman's rank-order correlation coefficient (rho); Mann-Whitney test, or Kruskal-Wallis test with a Dunn's post-hoc analysis). 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); and with GraphPad Prism Software version 5 (GraphPad Software Inc, San Diego, CA, USA).

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 method 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 our RT-qPCR to detect the four highly homologous CK transcripts.

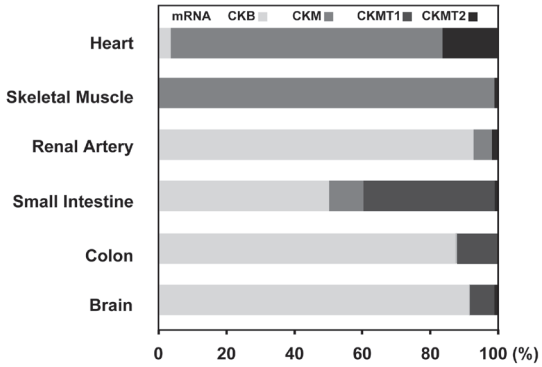
Subsequently, we assessed the human resistance arteries with these validated isoenzyme transcripts. Normalized CK mRNA copy numbers of the vascular tissue and the correlation with systolic and diastolic blood pressure are depicted in Figure 2, showing the strong correlation between CKB mRNA and blood pressure. Mean CKB mRNA copy numbers were around 90% higher in hypertensives compared to normotensives, respectively 19.3 (SE 2.0) vs 10.1 (2.1), p=0.0045. For the other isoenzymes, mRNA copy numbers in hypertensives compared to normotensives were for CKM, respectively 0.07 (0.02) vs 0.02 (0.01), 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. Non-parametric statistical methods did not significantly change the direction or the magnitude of the outcomes, with a Spearman's 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).

Table 2. Clinical characteristics of the participants

Clinical Parameter	Total group (n=13)	Normotensives (n=6)	Hypertensives (n=7)*
African Ancestry	6	2	4
Age (y)	42.9 (1.6)	41.2 (2.7)	44.3 (1.7)
Systolic blood pressure (mm Hg)	144.8 (8.0)	124.2 (4.5)	162.6 (10.4)
Diastolic blood pressure (mm Hg)	86.5 (4.3)	72.7 (3.3)	98.4 (3.1)
Heart rate (min^{-1})	79.2 (3.1)	70.8 (2.9)	86.4 (3.3)
Body mass index (kg/m^2)	25.9 (1.3)	24.8 (1.0)	26.9 (2.4)

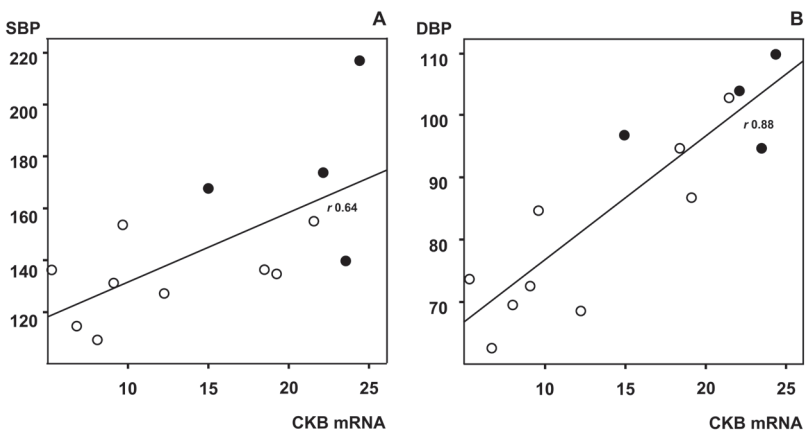
Data are expressed as mean (SE). *Hypertension was defined as SBP ≥140 or DBP ≥90 mm Hg, or the use of antihypertensive drugs. Three patients were diagnosed with stage 1 hypertension (SBP 140 to 159 or DBP 90–99 mm Hg), and four with stage 2 hypertension (SBP ≥160; or DBP ≥100 mm Hg). According to Dutch national guidelines, treatment of uncomplicated hypertension is imperative only at SBP ≥180 mm Hg.[15] Four hypertensives were treated, with ACE inhibitors, beta-adrenergic blockers, calcium channel blockers, or thiazide diuretics, as monotherapy or as combination therapy. None reached control.

Figure 1. CK isoenzyme mRNA in different human tissues



Validation of the creatine kinase (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 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).[9,16] 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. Importantly, this distribution pattern of tissue CK isoenzyme mRNA accords with previous reports on the distribution of tissue CK isoenzyme protein.[5,7,9,10,12]

Figure 2. Panel A and B. Correlation between human resistance artery CKB mRNA and blood pressure



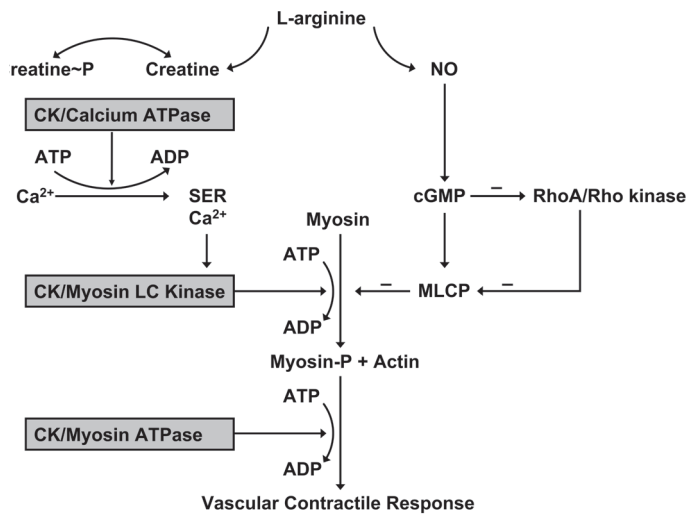
Correlation between human resistance artery creatine kinase (CK)-B mRNA and blood pressure. Scatter plot and least squares linear regression lines with Pearson product-moment correlation coefficients (r) for the association between blood pressure in normotensives and hypertensives (n=13) expressed in 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 normalized 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).

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

CK mRNA	Normalized copy number		Correlation Coefficient (r)	
	Mean	SE	SBP	DBP
CKB	15.00	1.91	0.64 (0.14 to 0.88)*	0.88 (0.64 to 0.96)*
CKM	0.05	0.01	0.61 (0.09 to 0.87)*	0.55 (0.00 to 0.84)*†
CKMT1	0.19	0.08	0.70 (0.24 to 0.90)*	0.33 (−0.29 to 0.74)
CKMT2	1.53	0.22	0.52 (0.04 to 0.83)*	0.70 (0.24 to 0.90)*

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 creatinekinase; 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 to 0.84), signifies (0.001 to 0.844).

Figure 3. CK and the main intracellular pathways of vascular smooth muscle contraction



This is a schematic representation of the main intracellular regulatory pathways of vascular smooth muscle contraction, based on Brewster et al.[5,6] Creatine and nitric oxide (NO) share a common precursor in L-arginine. Creatine kinase (CK) is colocalized with Ca^{+2}-ATPase and myosin ATPase, and evidence suggests the enzyme is also colocalized with myosin light chain (LC) kinase, to rapidly supply these enzymes with ATP using creatinephosphate (Creatine~P). NO, RhoA/Rho kinase, and calcium-dependent pathways are intracellular effectors of blood pressure-regulating systems that converge on metabolic processes fueled by CK.[5-7,19,22,23] Thus, high CK activity might lead to greater vascular contractility, partly through a lack of bioavailability of L-arginine for nitric oxide synthesis.[6,19] cGMP, guanosine cyclic 3',5'-(hydrogen phosphate); MLCP, myosin light chain phosphatase. SER, sarcoendoplasmic reticulum.

Discussion

We found a strong association between human resistance artery CK mRNA and systemic systolic and diastolic blood pressure, across the clinical spectrum of normotension and hypertension. We also provide a detailed method to simultaneously assess 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 multi-ethnic population, with an adjusted blood pressure increase of 7.98 [3.27 to 12.68] systolic and 4.69 [1.88 to 7.50] mm Hg diastolic per log CK increase;6 this was replicated in case control and independent population studies.[17,18] Furthermore, we reported that human isolated resistance artery contractility depends on CK, and that specific CK inhibitors greatly attenuate human vascular contractility in vitro.[19] 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.[6] 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 believe this is consistent with other physiological observations concerning 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 based on 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, vs 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 due to 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 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 Ca^{2+} and initiated by phosphorylation of the serine 19 residue of the myosin regulatory light chain (MLC) by a specific Ca^{2+} calmodulin-MLC kinase complex. This MLC 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 nitric oxide (NO) biosynthesis, through reducing bioavailability of L-arginine. Creatine and NO are both synthesized from L-arginine, but creatine synthesis demands nearly 10 times 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 messenger RNA. However, no correlation with blood pressure was reported.[24] 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, 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 diastolic blood pressure, and are also highly correlated with systolic blood pressure, while CK mRNA expression nearly doubles with hypertension.

This is in line with previous findings of CK as a main denominator of blood pressure,[6,17,18] and reports of significant vasodilation of isolated resistance arteries after CK inhibition.[19]

Furthermore, 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.[19,24] Yet, because of the large effect size expected, this sample size was calculated to be sufficient for the primary outcome. Another limitation is that we, and other researchers assessing mRNA expression levels, use the results as a proxy for functional differences that occur at the protein level, although mRNA levels 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 to 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 are needed 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.

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 to 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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Disclosures

LMB is an inventor on NL patent WO/2012/138226 (filed).

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Chapter 4

The effect of creatine kinase inhibition on contractile properties of human resistance arteries

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Abstract

Background: Creatine kinase (CK) is a main predictor of blood pressure, and this is thought to largely depend on high resistance artery contractility. We previously reported an association between vascular contractility and CK in normotensive pregnancy, but pregnancy is a strong CK inducer, and data on human hypertension are lacking. Therefore, we further explored CK-dependency of vascular contractility outside the context of pregnancy, in normotensive and hypertensive women.

Methods and Results: Nineteen consecutive women, mean age 42 years (SE 1.3), mean systolic/diastolic blood pressure respectively 142.6 (SE 5.9)/85.6 (3.4) mm Hg (9 hypertensive), donated an omental fat sample during abdominal surgery. We compared vasodilation after the specific CK inhibitor DNFB (10^{-6} mol/l) to sodium nitroprusside (10^{-6} mol/l) in isolated resistance arteries using a wire myograph. Additionally, we assessed predictors of vasoconstrictive force. DNFB reduced vascular contractility to 24.3% (SE 4.4), $p<0.001$, compared to baseline. Sodium nitroprusside reduced contractility to 89.8% (SE 2.3). Maximum contractile force correlated with DNFB effect as a measure of CK (r 0.8), and with vessel diameter (r 0.7). The increase in contractile force was 16.5 mN [9.1 to 23.9] per unit DNFB effect in univariable, and 10.35 mN [2.10 to 18.60] in multivariable regression analysis.

Conclusion: This study extends on our previous findings in pregnant normotensive women of CK-dependent microvascular contractility, indicating that CK contributes significantly to resistance artery contractility across human normotension and primary hypertension outside the context of pregnancy. Further studies should explore the effect of CK inhibitors on clinical blood pressure.

Introduction

We have reported that creatine kinase (CK, EC 2.7.3.2) is a main predictor of blood pressure in the general population [1]. The CK enzyme system serves as a conduit for high energy phosphoryl groups. Sequential phosphotransfers through the enzyme are responsible for transmission of ATP from mitochondria to ATP-consuming sites and maintenance of ATP/ADP ratios near the ATPases. At the ATP consuming site, CK rapidly regenerates ATP from creatinephosphate, catalyzing the reaction:

$$MgADP + creatine\sim P + H+ \leftrightarrow MgATP + creatine$$ [1–5].

Thus, the enzyme may facilitate highly energy-demanding functions such as salt retention and vascular contractility, thus promoting hypertension [1,2,5–7].

The activity of the enzyme in plasma was reported to be strongly and independently associated with blood pressure in the general population, with an increase in systolic blood pressure of 8.0 mm Hg (95% CI, 3.3 to 12.7) mm Hg per log CK increase after adjustment for age, sex, body mass index, and ethnicity [1]. Similar outcomes were found in a replication study [8]. Furthermore, analysis of human resistance arteries showed a high correlation between microvascular CK gene expression and clinical blood pressure (R=0.9) [6]. Finally, in pregnant normotensive women, vascular contractility was found to be highly CK-dependent [7]. However, the CK system undergoes profound qualitative as well as quantitative changes during gestation [3], and more work is needed to further characterize the role of this enzyme in pressor responses as despite the accumulating data indicating its clinical relevance, there is no direct evidence that the enzyme affects resistance artery function in normotension and primary hypertension outside the context of pregnancy. Therefore, we assessed the role of CK in contractility of isolated human resistance arteries from non-pregnant normotensive and hypertensive women.

Methods

Participants

Protocols were in accord with institutional guidelines and approved by the local institutional review board. The study was performed conform the declaration of Helsinki. Consecutive women of self-defined European or African ancestry undergoing an abdominal procedure for uterine fibroids with or without high blood pressure were eligible for inclusion. We excluded patients with secondary hypertension, patients with a history of cardiovascular events (angina pectoris, myocardial infarction, or stroke), patients who smoked, patients with vasculitis, diabetes mellitus, or other endocrine disorders; HIV infection; infectious hepatitis; malignancies, or bleeding disorders. All participants gave written informed consent.

Outcomes

The primary outcome was the extent of vasodilation induced with the specific CK blocker 2,4-dinitro-1-fluorobenzene (DNFB), as compared to baseline. The secondary outcome was the difference in vasodilation induced by DNFB, versus vasodilation with the NO donor sodium nitroprusside (SNP). Other, hypothesis-generating outcomes included the effect of bradykinin, predictors of maximum contractility, and outcomes based on blood pressure categories.

Sample size calculation

We based our sample size calculation on previous data [7], and we conservatively estimated to find a 65% vasodilation (35% residual contractility) compared to baseline with DNFB 10^{-6} mol/L as a primary outcome, with an σ of 15, needing 6 patients. In the secondary outcome we expected vasodilation to 25% (75% residual contractility) with sodium nitroprusside (SNP) at 10^{-6} mol/L, with an σ of 15, and we calculated that a total number of 19 persons needed to enter the study to detect this within-person difference with one-tailed $\alpha=0.05$ and $1-\beta=0.80$.

Blood Pressure

Blood pressure was measured with a Datascope Accutorr Plus monitor (Datascope Corp., New Jersey, USA), during the pre-operative assessment one month before the surgery, after 5 minutes of rest with the subject in the sitting position, using an appropriately fitted cuff on the non-dominant arm supported at heart level. 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-Specificity of DNFB

DNFB in the micromolar range is a specific CK inhibitor that forms a covalent derivative with a single cysteine residue to inactivate CK, inducing a rapid depletion of ATP while creatine phosphate is preserved [9–16]. Although DNFB is an amino and sulfhydryl group reagent that could react with many targets, and aspecific effects may occur at very high (millimolar or higher) dose ranges, when appropriately dosed in the micromolar range, the effect of DNFB is CK specific [9,11,12,14,16]. Micromolar DNFB typically inhibits both the forward and the reverse reaction of cytoplasmic as well as mitochondrial CK stoichiometrically [9], and the effect of DNFB is used to estimate tissue CK activity [10]. Importantly, the inhibition of CK activity is immediate and dose-dependent, at around 40% at DNFB 10^{-6} mol/l, and 55% at 3.10^{-6} mol/l, to 90% at 10^{-5} mol/l [9]. DNFB does not affect the intrinsic ability of myofibrils to develop tension, and tissues retain the ability for respiration and responses to ATP [13,14]. CK repletion completely reverses the inhibitory effect of DNFB, and muscle contractility and function is restored [13].

Microvessels' Preparation and Tension Measurements

The procedure was described previously [6,7]. In brief, after omental biopsy, performed at the start of the surgical procedure, 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, $NaHCO_3$ 24.8, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2, EDTA 0.26, and HEPES 50. Vessels were dissected under a microscope and cleaned of adherent adipose and connective tissues. Segments of resistance-sized arteries (~200 to 400 µm normalized internal diameter) were cut into 2-mm-long rings (2 to 4 per participant) that were mounted on 40-µm stainless steel wires in a Mulvany-Halpern myograph (Danish Myo Technology, Copenhagen, Denmark). The myograph bath contained PSS at 37°C bubbled with 5% CO2 and oxygen 95%, to maintain a pH of 7.4. The caliber of the vessel and the settings for distension were calculated using standard methods for wire myography [7]. We included vessels that were able to generate active tension against at least 50 mm Hg equivalent pressures. Each ring was normalized and set to the optimal diameter for active tension at 90% of the diameter of a passive (relaxed) vessel at a transmural pressure of 100 mm Hg. To assess integrity of the endothelium, vessels were contracted with noradrenaline (10^{-5} mol/L) and if relaxation to bradykinin (10^{-6} mol/l) was greater than 70% of the contraction the vessels were considered to be endothelium-intact. Maximum contractility was induced in the isolated arteries in duplicate with noradrenaline (10^{-5} mol/L) in KCl (125 mmol/L) substituted PSS (KPSS-NE). We also used KPSS-NE to reach a stable contraction during the long lasting experiment. We studied two main pathways of vasodilation after maximum contractility, inhibition of CK-dependent contractility and stimulation of the NO/cGMP pathway (Figure 1). First, the effect of SNP (10^{-9} to 10^{-4} mol/L) and bradykinin (10^{-10} to 10^{-6} mol/L)-induced vasodilation were studied, and lastly, the specific CK blocker DNFB (10^{-7} to 10^{-5} mol/L) was added. All concentrations refer to final bath concentrations.

Chemicals

All chemicals were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, USA.

Data Analysis

Data from multiple rings from the same subject were averaged. Residual contractility was calculated as the fractional decrement in contractile response after the addition of a vasodilator. Based on the dose finding analysis in our previous study [7], and the specificity studies of DNFB [9–16], we used the efficacy of the vasodilators at 10^{-6} mol/L for the main analysis. As DNFB is reported to stoichiometrically and specifically inactivate CK [9–16], residual contractility after DNFB 10^{-6} mol/L was used as a measure of intravascular CK activity as previously described [7,9,10]. Since the CK distribution is known to be skewed to the right [21], we expected a skewed distribution for the residual contractility after DNFB, and planned to perform a logarithmic transformation to base 10 with these and other skewed data, to achieve a more symmetric distribution. We used a paired Student's t-test to assess the primary outcome of vasodilation after DNFB compared to baseline. As a secondary outcome, we compared DNFB with SNP

mediated vasodilation in a paired t-test. Finally, as hypothesis-generating outcomes, we calculated the Imax and pIC50 (the negative logarithm to base 10 of IC50, which is the concentration leading to half-maximal inhibition) of DNFB, SNP, and bradykinin; and one-tailed Pearson's correlations between maximum resistance artery contractility and potentially predictive parameters such as the extent of vasodilation with different inhibitors, vessel size, and clinical characteristics including ethnicity, blood pressure, and body mass index (BMI), before entering variables with correlating significantly at $p \leq 0.05$ into multivariable regression analysis to further quantify the independent association with maximum vascular contractile force, using forced entry. Numerical ranges in square brackets are 95% confidence intervals. For the primary and secondary outcomes, the p value was one-tailed as there was pre-existent evidence on the direction of the outcome. For all other, hypothesis-generating outcomes, we conservatively used a two-sided p value of 0.05 or less to indicate statistical significance. Statistical analyses were performed with SPSS statistical software package for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinical Characteristics and Blood Pressure

Table 1. Clinical characteristics of included participants

			Uncontrolled Hypertension	
Parameters	**Total Group**	**Normotension**	**Untreated**	**Treated**
N / African ancestry	19 / 11	10/ 5	5/ 3	4/ 3
Age, years	42.4 (1.3)	40.2 (1.9)	44.8 (1.0)	44.5 (3.1)
Heart rate, min−1	82.0 (3.0)	75.3 (4.4)	85.0 (5.3)	90.8 (3.6)
SBP, preoperative, mm Hg*	143 (6)	127 (3)	146 (4)	174 (16)†
SBP on surgery day, mm Hg‡	126 (6)	115 (4)	129 (11)	148 (19)§
DBP, preoperative, mm WHg*	86 (3)	74 (3)	94 (4)	102 (3)†
DBP on surgery day, mm Hg‡	74 (3)	67 (4)	82 (7)	82 (6)§
Body mass index, kg/m2	25.9 (1.3)	24.5 (0.9)	24.7 (3.2)	31.0 (4.0)

Legends

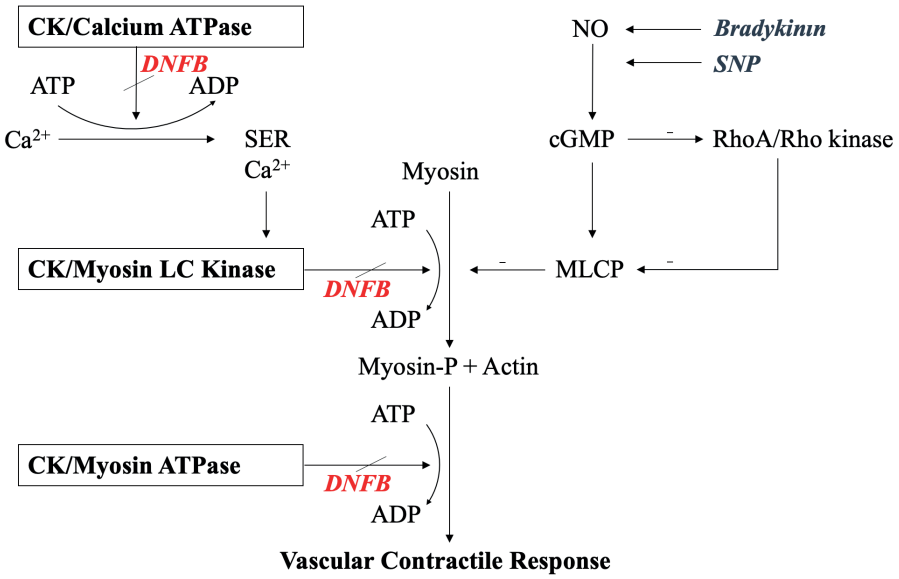
Data are mean (SE), unless specified otherwise; Based on blood pressure status, patients were American Society of Anesthesiologists Physical Status (ASA PS) class I–III.

Abbreviations: DBP, diastolic blood pressure; SBP, systolic blood pressure.

*Hypertension was defined as a blood pressure ≥140 mm Hg systolic (SBP) or ≥90 mm Hg diastolic (DBP), or the use of antihypertensive drugs. †Patients with treated hypertension had higher blood pressure levels than untreated hypertensives, as according to national guidelines [22] treatment of uncomplicated hypertension is only imperative at SBP >180 mm Hg. ‡Preoperative blood pressure measured in the supine position, 1 to 3 hours before the omentum biopsy, and after the use of standard preoperative sedative drugs. §With preoperative sedative and antihypertensive drug treatment.

Nineteen consecutive women participated in the study. The clinical characteristics are depicted in Table 1. In this study group, 11 out of 19 women were of African descent, and 9 were hypertensive (6 African-Dutch), reflecting the greater occurrence of fibroids and hypertension in women of African descent [23]. Only 4 hypertensives were treated, with angiotensin-converting enzyme inhibitors, beta-adrenergic blockers, calcium channel blockers, or thiazide diuretics, as monotherapy or as combination therapy. None reached control. The 5 untreated hypertensives (3 with stage 1 hypertension, and

Figure 1. Intracellular pathways of vascular smooth muscle contraction



This is a schematic representation of the main intracellular regulatory pathways of vascular smooth muscle contraction and mechanisms of action of vasodilators used in this study, based on Brewster et al [1]. Creatine kinase (CK) is depicted to rapidly regenerate ATP near ATPases. Calcium-dependent signalling pathways, as well as NO, cyclic GMP, and RhoA/Rho kinase pathways converge on metabolic processes fuelled by CK [1–7,9,17–20]. In this study vasodilation was achieved with the specific CK inhibitor dinitrofluorobenzene (DNFB), or through stimulation of NO-dependent pathway with sodium nitroprusside (SNP) and bradykinin. SER, sarcoendoplasmic reticulum; Mysosin LC Kinase, myosin light chain kinase; cGMP, guanosine cyclic 3', 5'-(hydrogen phosphate); MLCP, myosin light chain phosphatase.

2 with stage 2 hypertension), had never received antihypertensive drugs, although 2 had previously been diagnosed with hypertension by a medical doctor. Women with treated uncontrolled hypertension had a higher body mass index than normotensives and never-treated hypertensives, as well as the highest mean systolic and diastolic blood pressure, as per national hypertension treatment protocol, in the absence of additional risk factors, family doctors in the Netherlands are not compelled to start treatment in patients with uncomplicated hypertension unless SBP is >180 mm Hg [22].

Vessel Characteristics

We assessed two to three, 2 mm long artery segments per participant, at 90% of the diameter of the passive segment under a standardized transmural pressure of 100 mm

Table 2. Resistance artery characteristics by blood pressure status

Parameters	Total Group	Normotension	Uncontrolled Hypertension	
			Untreated	Treated
N	19	10	5	4
Microvessel Diameter, µm*	395.6 (22.9)	368.3 (29.2)	457.0 (55.7)	387.1 (34.8)
Maximum Force, mN	11.6 (1.3)	11.0 (1.9)	14.4 (3.0)	9.3 (2.0)
Force/100 µm vessel diameter	2.7 (0.2)	2.7 (0.3)	3.1 (0.4)	2.4 (0.4)
DNFB residual force, %†	24.3 (4.4)	23.1 (5.1)	33.3 (13.4)	16.4 (2.7)
Bradykinin residual force, %†	89.6 (2.0)	91.8 (3.2)	86.0 (3.5)	88.4 (2.4)
SNP residual force, %†	89.8 (2.3)	87.0 (3.8)	91.8 (2.0)	94.5 (4.6)

Legends

All arteries were assessed at an estimated transmural pressure of 100 mm Hg. Data are mean (SE), unless specified otherwise. There were no significant differences in vessel characteristics based on clinical blood pressure status in this standardized test (p values ranging from 0.29 to 0.65). *Microvessel internal diameter. †Residual contractile force with 10−6 mol/L of the vasodilator. Abbreviations: DNFB, dinitrofluorobenzene; SNP, sodium nitroprusside.

Hg. Vessels were resistance artery-sized, with a mean internal diameter of 395.6 µm (SE 22.9). At this standardized pressure, we found no significant differences in vessel characteristics between blood pressure categories as a tertiary, hypothesis-generating outcome (Table 2).

Inhibition of Vascular Contractility

The vascular contractile force was reduced to 24.3% (SE 4.4, $p<0.001$) after DNFB 10^{-6} mol/L, as the primary outcome. Residual contractility after SNP 10^{-6} mol/L was 89.8% (SE 2.3), a difference of 65.5% [54.9 to 76.1%], $p<0.001$ with DNFB (Figure 2). Residual force after bradykinin was similar to SNP, 89.6% (SE 2.0). The I max and pIC50 of these vasoactive agents were respectively 87.44 (SE 1.94) and 6.55 (0.08) for DNFB; 21.52 (2.92) and 6.09 (0.12) for SNP; and 11.26 (2.19) and 7.78 (0.11) for bradykinin. Thus, the relative potencies were in the order bradykinin>DNFB>SNP. In line with the presumed mode of action depicted in Figure 1, with CK-dependent contractility as the final common step, we found large differences in vasodilation response, with DNFB showing the greatest inhibitory efficacy (the maximal inhibitory effect of SNP and bradykinin was respectively 24.6 and 12.9% of DNFB effect).

Predictors of the Maximum Contractile Response

In the correlation analysis, only DNFB effect and vessel diameter were significantly

Figure 2. Vasodilation on isolated resistance arteries

We stimulated the NO-dependent pathway with (A) sodium nitroprusside (SNPW) or (B) bradykinin, and compared the effect on maximum contractile responses with (C) the specific creatine kinase (CK) inhibitor dinitrofluorobenzene (DNFB). P<0.001 for the difference between DNFB and SNP. -Log concentration (mol/L).

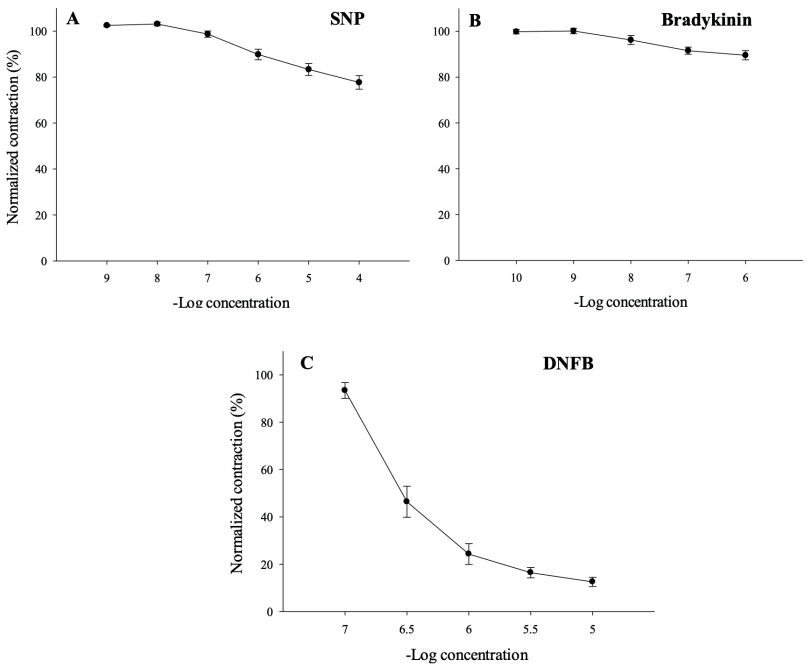
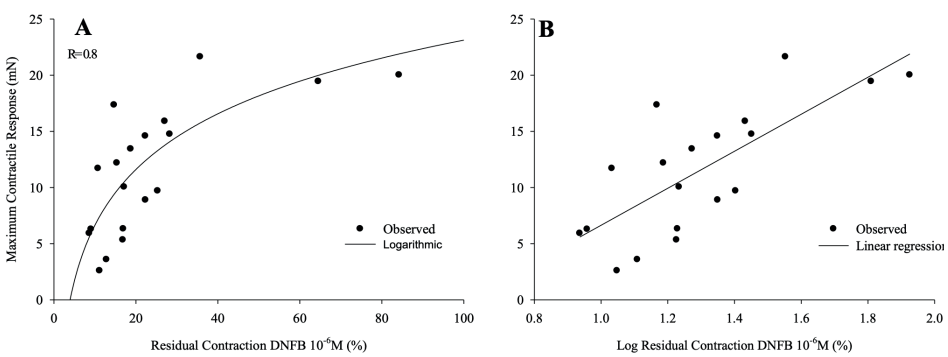


Figure 3. Correlation between residual contraction after DNFB and maximum contractile response

Association between the maximum resistance artery contractility and CK activity assessed by DNFB. Panel A shows the fitted logarithmic curve and panel B the linearized curve of the association between DNFB effect and maximum contractility; p<0.001.



associated with the maximum contractile response, with a correlation coefficient of respectively 0.76 and 0.74 ($p<0.001$); Figure 3. In contrast, the correlation coefficient was 0.02 for both bradykinin and SNP ($p=0.46$). Other parameters as mentioned in the Methods section did not significantly correlate with maximum contractility (values 0.00 to 0.18, values 0.23 to 0.50; data not shown).

We further quantified the association between the CK inhibitor, vessel size and maximum contractile response in univariable regression analysis. Vascular contractile force increased 16.48 [9.09 to 23.87] mN per unit increase in DNFB effect (log residual contractility after DNFB 10^{-6} mol/L, as a proxy for CK activity), and 4.27 mN [2.31 to 6.24] per 100 µm increase in vessel diameter.

Finally, in multivariable regression analysis, the multiple correlation coefficient (R) was 0.83, with an square of 0.69, an adjusted square of 0.65, and a standard error of estimate of 3.39; thus 65% of the variance in vessel contractility was predicted by measures of CK and vessel diameter. The beta-coefficients were respectively 10.35 [2.10 to 18.60] for estimated CK and 2.58 [0.42 to 4.72] per 100 µm vessel diameter, without significant interaction (=0.95). This indicates a strong independent association of CK and vessel size with vascular contractility.

Discussion

In this paper we extend on our findings of CK as a main determinant of vascular contractile force in normotensive pregnancy [7], to resistance artery contractility outside the context of gestation. During pregnancy, the CK enzyme is highly induced, in particular in smooth muscle [3]. Hence we studied, to our knowledge for the first time, the effect of direct CK inhibition on isolated resistance arteries from non-pregnant normotensives and treated and never-treated stage 1 and 2 primary hypertensive subjects, under standardized in vitro conditions. Our data indicate that the CK phosphoryl transfer system has a strong contribution to resistance artery contractility in humans across the spectrum of normotension to hypertension. The results are consistent with animal [24], case control [25], and population studies [1,8] on CK and blood pressure, which indicated CK is relevant for pressor responses.

CK is central to a spatially arranged intracellular enzymatic network, which also includes adenylate kinase, carbonic anhydrase and glycolytic enzymes, that functions to support high-energy phosphoryl transfer, such as in smooth or striated muscle contraction, and signal communication between intracellular ATP-generating and ATP-consuming/ATP-sensing processes [1–7].
The association of CK with blood pressure is present across the blood pressure spectrum, and is reported to concern enhanced ATP buffer capacity and attenuated NO-mediated functions. High intracellular CK activity, whether constitutive, induced, or both, may rather directly enhance contractile responses by enhancing cellular energy and contractile reserve, thus promoting hypertension. In addition, CK-dependent microvascular structural alterations including trophic responses of the artery wall and rarefaction of

the vascular bed (associated with skeletal muscle, Type II fiber predominance, and high resting serum CK), as well as greater CK-dependent renal sodium retention are thought to contribute to higher blood pressure levels with high CK [1,2,5–7,26,27].

Regarding vascular contractility, as previously reported by us and others, CK acts as an energy transducer at the vascular smooth muscle contractile proteins, supplying ATP for the contractile process as a final and rate limiting step at myosin ATPase (Figure 1) [1–7]. Vascular smooth muscle contraction is thought to consist of a fast, force-generating component at relatively high-energy costs, and a slow, tonic ADP-dependent maintenance of tension [1–7]. With greater CK activity, ADP levels at the contractile proteins may not reach the level required for tonic maintenance of tension, leading to excessive vascular contractility, delayed relaxation, and enhanced pressor responses [1,2,5–7].

The subsequent work of our group and others has resulted in experimental, clinical, and population data indicating that CK might enhance pressor responses [1,2,5–8,24–27]. CK gene expression in human resistance arteries was found to have a near perfect correlation with clinical blood pressure levels (R 0.9 [0.6 to 1.0]) [6]. In line with these findings, a strong association was reported between blood pressure levels and plasma CK at rest, in population studies of White European, South Asian, as well as African subgroups [1,8]. We also found a high risk to develop hypertension in a retrospective analysis of white men with idiopathic hyperCKemia [25]. Furthermore, it was shown in animal models, that high CK activity in cardiovascular tissue precedes the development of hypertension, further increases during hypertension, and lowers upon effective antihypertensive drug therapy [24,28,29]. Finally, resting plasma CK activity was found to be the main independent predictor of failure of antihypertensive therapy [27]. However, hitherto, direct evidence was lacking that CK affects microvascular contractility in human hypertension outside the setting of gestational induction of CK [3].

The main strength of this study is that we now provide evidence that the contractile response of resistance arteries of non-pregnant human normotension as well as treated and never-treated primary stage 1 and 2 hypertension is highly CK-dependent; with an efficacy of the CK inhibitor as a vasodilator that is higher than of known NO-dependent vasodilators used in the same protocol. This implies that rapid ATP regeneration near ATPases is relevant for vascular function across the blood pressure spectrum.

Conversely, some may suggest that the general anesthesia used when procuring the human samples might have affected vascular contractility, but it is unlikely that the anesthetic would have affected vessels differently, and we reported similar inhibitory effect of DNFB on omental vessels from pregnant women receiving epidural anesthesia [7].
Sixteen included women were either normotensive or had been diagnosed with hypertension by a doctor previously. However, theoretically, we could have falsely classified the remaining 3 women as hypertensive as we used only one blood pressure measurement. This is a limitation of this study. On the other hand, even single standardized clinical blood pressure readings are relevant and strongly related to mortality [30]. Furthermore, the main and secondary outcomes were analyzed as a

continuous measure, without categorizing blood pressure levels.

All women had fibroids, and our findings could be related to, or exaggerated by this condition. The presence of uterine fibroids is known to be associated with greater hypertension risk, and this may involve CK [23]. However, as the association between CK and blood pressure does not depend on the presence of uterine fibroids, as it has also been described in the general population [1,8], and in men [25].

A limitation of the study is the small sample size typical for studies on small arteries in humans [31,32] as the tissue harvesting requires a surgical procedure. The sample size was sufficient for the primary outcome, indicating a robust effect of CK inhibition on vascular contractility across all blood pressure categories, but limits statistical analysis in subgroups including blood pressure status.

This study was designed to assess the dependency of resistance artery contractile responses on CK, using the gold standard of in vitro assessment of vascular contractility under standardized isometric circumstances at a "normotensive" equivalent pressure of 100 mm Hg for all vessels studied [31–35]. With standardized measurements at 100 mm Hg, the main limitation of the method is that it impedes inferences regarding vessel responses based on clinical blood pressure categories (up to 220 mm Hg in these patients), and the results do not reflect or consider vessel characteristics or total energy expenditure at higher pressures [19,32,33]. The total energy expenditure of resistance arteries is reported to depend on blood pressure levels [6,19,28]. This probably reflects the in vivo demands of contractility and active stiffening against higher blood pressure levels than measured in our study, in changing circumstances that are neither continuously isometric, isobaric, or isotonic [6,19,28,31–35]. Also, high tension with increased stiffness is associated with increased turbulence in vivo, which exacerbates energy costs, but we do not model turbulence in our study. In addition, in established hypertension, structural vascular abnormalities are found, including vessel wall remodeling and rarefaction [19,31–35]. Both adaptations are associated with enhanced CK activity [1,2,26,27]. Thus, existing evidence indicates that ATP-demanding functions such as narrowing, stiffening, and reduced relaxation of the resistance artery wall may increase vascular energy expenditure in hypertension [1–8,19,25–28,31–35]. Our proof-of-principle data of CK-dependency of resistance artery contractility provide compelling evidence to support further, larger, studies to evaluate energy expenditure of human vascular smooth muscle at higher pressures.

In summary, in the present work we extend on previous population-level, clinical, and experimental evidence of the potential role of CK in human pressor responses. We had showed that CK is the main independent predictor of blood pressure, and of failure of antihypertensive therapy [1,2,6,27]. In addition, CK inhibition reduced resistance artery contractility in pregnant normotensive women [7]. As gestation is a strong inducer of smooth muscle CK, we further characterized the role of the enzyme in resistance arteries in human normotension and hypertension outside the context of pregnancy, measured under standardized test conditions. Our results indicate that across the spectrum of human normotension to hypertension, CK activity is a major determinant of microvascular

contractility. This work might help initiate exploration into the development of CK inhibitors as means to reduce blood pressure in hypertensive patients.

Acknowledgments

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Conflict of Interest

L.M. Brewster is an inventor on NL patent WO/2012/138226 (filed).

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Chapter 5

Creatine kinase inhibition lowers systemic arterial blood pressure in spontaneously hypertensive rats: a randomized controlled trial

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Abstract

Objective: Creatine kinase is reported to be a main predictor of blood pressure in the general population, with a strong correlation between resistance artery creatine kinase expression and clinical blood pressure in humans. The enzyme rapidly regenerates ATP near cytoplasmic ATPases involved in pressor responses, including resistance artery contractility and renal sodium retention. Therefore, we assessed whether creatine kinase inhibition reduces blood pressure.

Methods: We implemented the "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) guideline. In a 4-week randomized controlled trial, male 16-weeks-old spontaneously hypertensive rats (N=16) were randomly assigned to the specific competitive creatine kinase inhibitor beta-guanidinopropionic acid (3%) supplemented chow vs. standard chow. Blood pressure measured by the tail-cuff method was the main outcome. Other outcomes included vasodilation in isolated arteries and renal renin expression.

Results: Creatine kinase inhibition reduced blood pressure safely and reversibly. Mean baseline blood pressure of respectively 191.5 (SE 4.3) systolic and 143.1 (4.1) mm Hg diastolic was reduced by respectively 42.7 (5.5) systolic and 35.6 (5.0) mm Hg diastolic ($p<0.001$) compared to controls, with evidence of enhanced vasodilation and a diuretic effect.

Conclusion: To our knowledge, this is the first report on the blood pressure lowering effect of creatine kinase inhibition. Our data indicate that modulation of the creatine kinase system is a potential novel treatment target for hypertension.

Key words: antihypertensive treatment, betaguanidinopropionic acid, blood pressure, creatine kinase, drug development, hypertension, spontaneously hypertensive rat

Abbreviations: AGAT, L-arginine:glycine amidinotransferase; ARRIVE, Animal Research Reporting of In Vivo Experiments; CK, creatine kinase; DNFB, dinitrofluorobenzene; GAMT, N-guanidinoacetate methyltransferase; GPA, beta-guanidinopropionic acid; L-NNA, N-omega-nitro-L-arginine; SHR, spontaneously hypertensive rat

Introduction

Creatine kinase (CK, EC 2.7.3.2) is reported to be a causal factor in primary hypertension.[1-7] The enzyme catalyses the rapid and reversible transfer of a phosphoryl group from creatine phosphate to ADP, thereby forming creatine and ATP:[1-9]

$$\text{Phosphocreatine} + \text{MgADP} \leftrightarrow \text{Creatine} + \text{MgATP}$$

Resting plasma CK activity was reported to be a main predictor of blood pressure in random population samples.[2,4] Although men, the obese, and persons of West-African ancestry have higher mean tissue and plasma CK activities,[9-12] the association with blood pressure was independent of age, sex, body mass index, and ethnicity.[2] Further studies indicated that plasma CK was strongly associated with failure of antihypertensive therapy,[13] and human resistance artery contractility was found to be highly CK-dependent,[3] while CK mRNA of human resistance arteries showed a near-perfect correlation with clinical blood pressure.[5] Importantly, high CK preceded the development of hypertension, in animals[7] and humans.[6,9]

Evidence indicates that CK enhances vascular contractility and increases blood pressure through rapid regeneration of ATP near cytosolic ATP-utilizing enzymes involved in pressor responses, including myosin light chain kinase and myosin-ATPase at contractile proteins, and Ca^{2+}-ATPase at the cellular membrane.[1-8] In addition, the enzyme promotes sodium retention, as it regenerates ATP near Na^{+}/K^{+}-ATPase in the basolateral membrane of the renal tubular cell, which drives sodium retention throughout the kidney.[1,8,14] Also, the demand of L-arginine for creatine synthesis is thought to lower nitric oxide (NO) bioavailability.[2,3]

Taken together, the existing evidence supports a potential role for CK and enhanced ATP buffer capacity in hypertensive disorders. Therefore, we explored whether CK inhibition with the creatine analogue and competitive CK inhibitor beta-guanidinopropionic acid (GPA) reduces blood pressure in an animal model of hypertension and high CK activity,[2,7] the spontaneously hypertensive rat (SHR).

Methods

ARRIVE guidelines

We used the ARRIVE (Animal Research: Reporting of in Vivo Experiments) guidelines to design the trial using the 3 R principle (replace, refine and reduce animals in research), and to improve the information published from our data, thereby minimizing unnecessary use of animals in future studies.[15]

Ethical Approval

The Animal Ethical Committee of the University of Amsterdam, the Netherlands, approved of all procedures described in this paper (Registry number DFC102100), which are in conformity with national[16] and European[17] legislation, and with the Federation of Laboratory Animal Science Associations (FELASA) recommendations.[18]

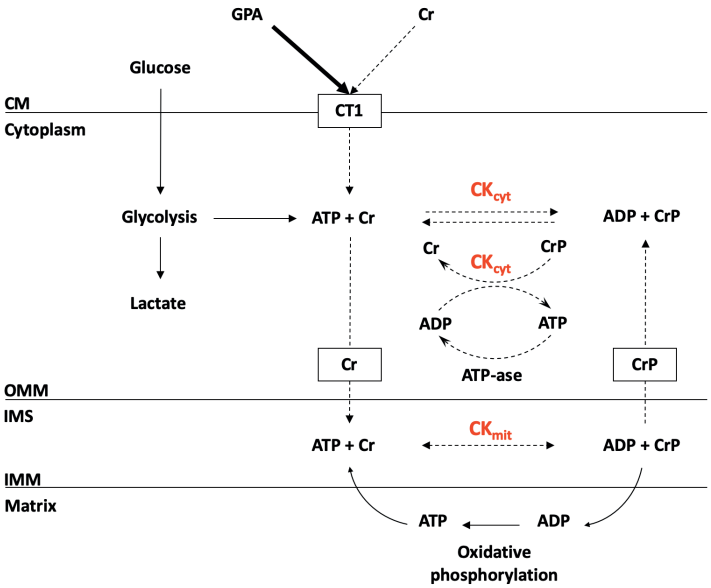
The competitive creatine kinase inhibitor beta-guanidinopropionic acid

GPA (or N-(aminoiminomethyl)-beta-alanine; $C_4H_9N_3O_2$), is a structural isomer and competitive inhibitor of creatine ($C_4H_9N_3O_2$). The flux through the CK reaction is linearly correlated to the intracellular concentration of creatine,[19] which is either absorbed in the intestine from dietary sources or synthesized de novo.[20,21] Creatine synthesis demands a substantial part of bioavailable L-arginine.[2] Kidney L-arginine:glycine amidinotransferase (AGAT, EC 2.1.4.1) catalyses the first, rate-limiting and committed step, of L-arginine and glycine to guanidinoacetic acid. Subsequently, liver S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2), methylates guanidinoacetic acid to produce creatine, which enters peripheral tissue through the saturable, sodium- and chloride-dependent creatine transporter 1 (CT1). A physiological constituent of mammalian blood, tissue, and urine,[20,21] GPA is synthesized by AGAT as well, from L-arginine and beta-alanine.[21] The estimated plasma concentration in healthy rats is 0.06 (SD 0.02) micromol/L[22] and clearance is probably renal, akin to other guanidino compounds.[20,21,23]

GPA inhibits the flux through the CK reaction, potentially through several pathways. It is a competitive and specific inhibitor of creatine absorption in the gut, and there is evidence that GPA also inhibits creatine synthesis by AGAT. However, the main effect is the efficient and competitive inhibition of cellular creatine uptake by CT1 (Figure 1), with a Ki of 8.8 to 120 micromol/L, rendering GPA effective in pharmacological doses; 1 to 3% in food used in most studies.[20,21] The resultant reduction in intracellular creatine reduces the flux through the cytoplasmic CK reaction linearly.[19] Furthermore, GPA can be transported into the cell by CT1, and phosphorylated by cytoplasmic, but not mitochondrial CK.[24] Both GPA and phosphorylated GPA are ''inefficient substrates'' for the CK reaction: in vitro Vmax values are <1% of the Vmax values of creatine and phosphocreatine.[20,24,25] This effect further reduces cytoplasmic phosphocreatine and CK-dependent ATP buffer capacity.[20,21] Finally, reduced intracellular creatine concentrations attenuate the intracellular non-enzymatic conversion to creatinine. Thus, a drop in plasma creatinine levels during GPA ingestion marks its efficacy in reducing intracellular creatine.[20,21]

Although not previously evaluated in hypertension, GPA has been studied extensively in animal models.[20,21] The modulated ATP buffer capacity is reported to result in a shift in skeletal muscle from high CK, predominantly glycolytic Type II muscle fibers, to low CK, predominantly oxidative Type I fibers. This apparent shift towards oxidative metabolism results in greater endurance capacity, enhanced glucose tolerance, and weight loss. Furthermore, greater resistance against brain ischaemia was observed.[20,21] In the

Figure 1. Cellular mechanism of action of beta-guanidinopropionic acid



Cytosolic creatine kinase synthesizes and transports creatine phosphate to ATPases including Na+/K+-, Ca2+-, and myosin ATPase, where it rapidly regenerates ATP in situ, maintaining a high ATP to ADP ratio near the ATPase. Mitochondrial creatine kinase synthesizes creatine phosphate from mitochondrial ATP creating a phosphoryl group shuttle towards the cytoplasm. Thus, the flux through the creatine kinase reaction is highly creatine-dependent. The main effect of beta-guanidinopropionic acid is to competitively inhibit cellular creatine uptake through the creatine transporter CT1 in the cellular membrane, thought to result in an attenuated flux through the creatine kinase reaction (broken lines).1–8,14,20,21 GPA, beta-guanidinopropionic acid; Cr, creatine; CM, cellular membrane, CKcyt and CKmit, cytoplasmic and mitochondrial CK; OMM and IMM, outer and inner mitochondrial membrane; IMS, intermembrane space.

unstressed heart of the intact animal, left ventricular systolic pressure, cardiac output, and rate of tension development were unchanged.[20]

During high workload, studies showed unchanged or reduced peak left ventricular developed pressure and cardiac output.[20]

GPA is sold on the internet for human use. Sportspersons tend to use it to increase stamina and reduce weight. We obtained beta-guanidinopropionic acid from Purebulk Vitamins and Dietary Supplements (Roseburg, Oregon, USA). Purity was found >99% by nuclear magnetic resonance analysis (VUMC, Division of Organic Chemistry, Department of Chemistry and Pharmaceutical Sciences, Amsterdam, the Netherlands). Cyanide, assessed because cyanamide is used in GPA synthesis, was below detection limits (<1 p.p.m.; Eurofins Omegam Laboratories, Amsterdam, the Netherlands).

Pilot studies

In accord with the 3 R principle, the ethical committee approved of pilot studies in

convenience samples of female and male SHR. The SHR is the main animal model of hypertension. In addition, SHR is reported to express high CK activity in the cardiovascular system, which occurs before the onset of hypertension.[2,7] We assessed dose, acute effects, potential side effects, reversibility of the effects, as well as technical, financial, ethical, organizational, and scheduling feasibility of the final study. Animal chow was obtained at A. Blok Animal Nutrition, Woerden, the Netherlands.

We found in 12 male rats that GPA 0.1 weight percent vs. control chow did not affect blood pressure. The potential acute (48 h) blood pressure-lowering effect of GPA 0.3 or 3% was assessed in 8 female rats, 4 age-matched rats in each intervention group, with a mean age of 51.5 weeks (SE 2.3). In addition, we assessed reversibility of blood pressure effects with chronic ingestion during 5 weeks, 3 weeks active treatment with 2 weeks follow up, in 6 age-matched female rats vs. 6 controls (mean age 31.1 weeks, SE 1.0). Animals were housed and taken care of as described below. Food was supplied ad libitum, with the intake, weight and blood pressure measured as detailed below, but no blood was drawn.

All animals in the pilot trials appeared healthy without apparent side effects of GPA. The last 5 blood pressure measurements out of 10 per assessment cycle appeared to yield the most stable readings, and these were used for the final analysis. In the acute study, systolic and diastolic blood pressure lowering occurred with GPA 3%. Assessed at 3,7,11, 24 and 48h, blood pressure lowering was first noted at 11 h and reached statistical significance at 48 h with mean SBP/DBP 170.9 (SE 3.5)/115.6 (5.3) in GPA vs. 198.2(8.4)/136.8(9.5) mm Hg in controls.

In a 3-week active treatment with GPA 3%, baseline SBP/DBP 184(6.1)/129(3.0) mm Hg with GPA vs. 188(8.2)/121(7.6) mm Hg in controls, lowered to SBP/DBP 165(2.8)/114(2.7) with GPA vs. 189(5.5)/135(5.6) in controls. Blood pressure returned to baseline values within 7 to 14 days after stopping GPA (SBP Day 7, GPA 181 vs. controls 190 mm Hg; Day 14, GPA 189 vs. controls 186 mm Hg, with similar diastolic responses).

In addition, the rats assigned to GPA significantly reduced their food intake in the first week of active treatment, to 55 (SE 3.3) g vs. 136 (2.7) g in controls ($p<0.001$). Accordingly, body weight of animals fed GPA reduced with 8% in the first week, 221 (5.9) to 205 (2.4) gram vs. 220 (7.6) to 235 (6.5) g in controls. Thereafter, the intake in the GPA group increased and was 95.2 (4.6) g with GPA vs. 137 (10.2) in controls at week 3, with a body weight of 221 (6.4) with GPA, vs. 234 (SE 6.4) g in controls ($p>0.05$).

Trial design

Based on our pilot data, we designed a 4-week randomized controlled trial with two parallel arms: GPA 3% added to a standard laboratory rodent diet, vs. control diet. The experimental unit was a single SHR, individually randomized after a 2 weeks' handling period to the intervention or control group using random numbers. Treatment allocation was concealed, but clinical investigators and outcome assessors were not masked, except for blood and tissue analyses.

Based on the results of our pilot studies, the animals in the final study were pair fed, with a reduced daily food supply in both the GPA and the control during the first 10 days of the trial of 15 gram, considered to be the minimum allowable by the clinical veterinarian. We planned to supply the food ad libitum after 10 days, as we noticed in the pilot studies that food intake in GPA-fed rats returned to normal after 1 week. The primary outcome was blood pressure. Other outcomes were heart rate (HR), body weight, plasma estimations including creatinine and cholesterol, heart weight and heart weight-to-body weight ratio, NO-synthesis-dependent and independent contractility responses in mesenteric arteries and aorta, ATP in heart and skeletal muscle, renal renin mRNA, and tissue creatine kinase isoenzyme mRNA. We used a fixed, a priori calculated sample size, based on our pilot study and existing data of blood pressure reduction in SHR with other antihypertensive drugs,[26] conservatively estimated to find a 16 mm Hg decrease (SD 7) in systolic blood pressure after 4 weeks of intervention, and calculated to need at least 6 rats in each group with a 2-tailed α=0.05 and 1-β=0.80.

Animals, Housing, and Husbandry

Animals were housed and cared for at our hospital's Animal Research Institute AMC in accord with the Dutch Act on Housing and Care for Research Animals, the European Union Directive on the protection of animals used for scientific purposes, and the FELASA recommendations.[16-18]

Fourteen-week-old male SHR (N=16, mean body weight 316.9 g) were obtained from Charles River (Maastricht, the Netherlands). The animals were individually housed in a cage with water ad libitum, and Lignocell S8/15 laboratory animal bedding (J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg, Germany) changed weekly. The temperature at the facility was maintained between 19 and 24 °C, with a humidity of 40 to 60% (both checked and registered daily), and a light cycle of 12 h light (maximum 350 lux) and 12 hour dark (7 pm to 7 am, without daylight saving time adjustment), in a sound reduced and ultrasound-free environment. Pathogens were actively monitored and controlled.[18] Food intake and weight were monitored daily, and the animals were checked daily throughout the experiment for behavioral, physical, or other health changes by Institute's staff under the supervision of a clinical veterinarian.

Blood Pressure Measurement

Standardized tail-cuff blood pressure measurements of conscious rats were performed after a 2-week acclimation period. Measurements were once weekly with the validated CODA system (Kent Scientific Corporation, Torrington, CT, USA), using a heating pad to obtain a body temperature of 35 to 37ºC. This device actually measures both the systolic and the diastolic blood pressure, using volume pressure recording sensor technology. A measurement cycle lasted 10–15 minutes (animal installation and warming: 5–10 min; acclimation cycles: 2 min; measurements: 30 seconds, with a 5 second delay between measurements, in total 3–4 min). During the measurements, care was taken to ensure minimal stress for the animals. Based on our pilot studies, we used the last 5 out of 10

measurements per cycle for our analyses.

Blood and Tissue Analyses

Blood for non-fasting biochemical estimations was drawn from the tail vein at baseline and day 28, using inhalation anaesthesia with isoflurane 2.5%. Blood was placed on ice immediately, centrifuged at 4º C, snap frozen in liquid nitrogen, and stored at -80 ºC until analysed. All plasma analyses were performed on a Modular Cobas 8000 (Roche Diagnostics, Darmstadt, Germany). At day 28, animals were anesthetised by a certified staff member of the animal facility with ketamine (90 mg/kg)-dexmedetomidine (0.125 mg/kg)-atropine (0.05 mg/kg) (KMA) through intraperitoneal injection. Aorta and mesenteric arteries were handled as described below. The brain, heart, liver, kidney, and m.quadriceps femoris were rapidly excised, immediately rinsed in phosphate buffered saline solution (PBS), snap frozen in liquid nitrogen, and stored at -80 ºC until analysed. The frozen heart and the lungs were weighed. The cortex of the right kidney was cut into 5 mm3 parts, and incubated overnight in RNAlater at 4 ºC, to be stored at -80 ºC until further use. ATP in heart and quadriceps muscle, kidney renin mRNA, and CK mRNA in brain, heart, skeletal muscle, kidney, and mesenteric artery were analysed as previously described.[5,27,28]

Artery Preparation and Tension Measurement

Third order mesenteric arteries were carefully excised and handled as described previously,[3,5] before transfer into myograph baths (Danish Myo Technology, Copenhagen, Denmark), at 37 ºC. Maximum contractility was induced in duplicate with norepinephrine (10^{-5}mol/L) in KCl (125 mmol/L)-substituted physiologic salt solution (KPSS) as previously described.[3,5] After washing and 20 minutes of rest, the vessels were preconstricted with phenylephrine (10^{-5}mol/L), adding methacholine (10^{-5}mol/L) to assess endothelial-dependent vasodilation. Hereafter, cumulative concentration-response curves for methacholine (10^{-9} to 10^{-4} mol/L), SNP (10^{-9} to 10^{-4} mol/L), and the specific and irreversible CK inhibitor dinitrofluorobenzene (DNFB),3 (10^{-6} to 10^{-3} mol/L) were constructed. To our knowledge, DNFB was not previously used in the SHR, a strain which is known to overexpress CK.[2,7] The effective concentrations of this CK inhibitor, which is suitable for vessel studies because of its immediate effect, were higher than we previously used in human resistance arteries, but in accord with some earlier animal vascular studies.[29]

The thoracic aorta was carefully excised and immediately placed in Krebs-Henseleit buffer (mmol/L) 118.5 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 1.2 KH_2PO_4 and 5.6 glucose at room temperature, aerated with carbogen, pH 7.4. The aorta was cut into 2 mm segments, mounted in an organ bath, containing 5 mL aerated Krebs buffer at 37°C, and attached to a force transducer. Readout was through a PowerLab data acquisition system (AD Instruments, Castle Hill, Australia). After 1 hour of equilibration at an isotonic resting tension of 10 mN, (maintained throughout the experiment), the segments were contracted twice for 10 min with a depolarizing high K^+ Krebs-Henseleit solution (with 40 mmol/L NaCl substituted by equimolar KCl) using intermediate washing steps of 20 min intervals. Subsequently, the vessels were pre-contracted with phenylephrine

(10^{-6} mol/L). After reaching a steady level of >60% contraction compared with previous K^+-induced depolarization contraction, methacholine (10^{-5} mol/L) was added to assess the endothelial integrity. When intact, 40 mmol/L K^+ was added after washing to obtain a maximal contractile response, and a cumulative concentration-response curve to methacholine (10^{-9} to 10^{-6} mol/L) was made after washing and 30 minutes re-equilibration. Finally, to assess the contribution of NO-dependent vasodilation, a segment was incubated with N-omega-nitro-L-arginine (L-NNA) (10^{-5}mol/L) during 30 minutes prior to the addition of methacholine (10^{-9} to 10^{-6} mol/L). All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Data Analysis

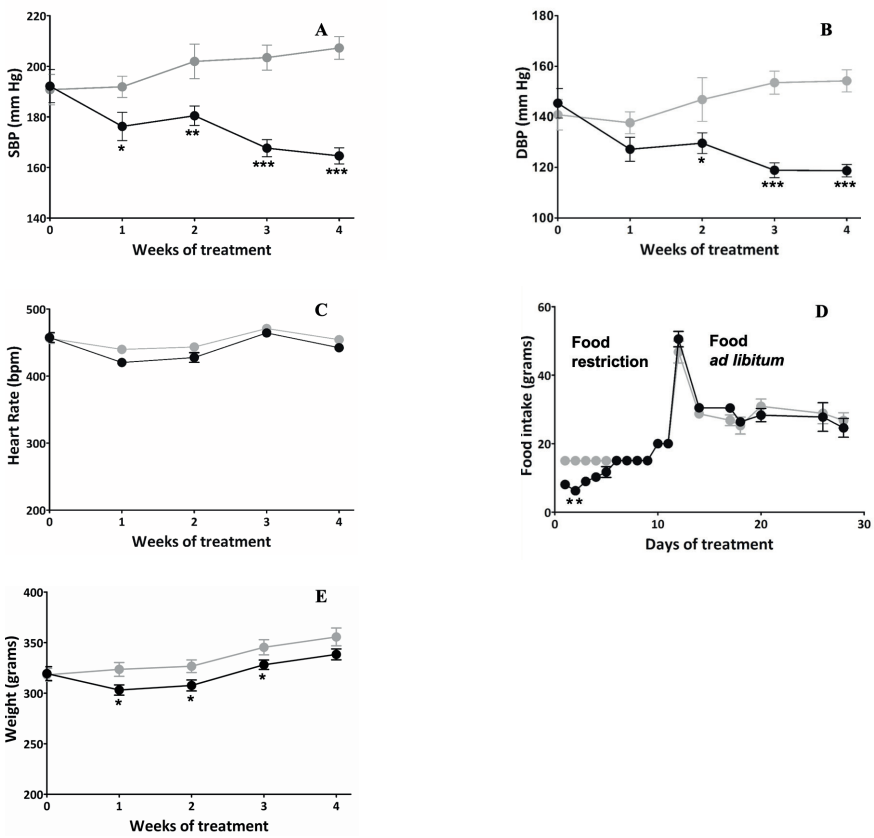
The primary analysis was intent-to-treat, with GPA vs. control rats as the primary unit of analysis. Because of the small sample size, the distribution of the data could not be formally tested. Since parametric analysis may not be accurate with small sample sizes, and nonparametric analysis may lack power to detect a significant difference, we used parametric statistics for our primary analysis (i.e. arithmetic mean with SEM, the unpaired t test, and one-way analysis of variance with the appropriate post-test with Bonferroni correction); and reanalyzed the data in a sensitivity analysis with non-parametric methods (i.e. median with interquartile range, Mann-Whitney test, or Kruskal-Wallis test with a Dunn's post-test). In addition, we conducted multivariable regression analysis to assess the predictors of blood pressure at week 4. For the primary outcome, we used a one-tailed p value, since we formulated a hypothesis on the direction of the outcome. We did not adjust the p values for multiple outcomes, but limited formal statistical testing on non-primary outcomes, and only used one-sided p values with pre-existent evidence or a hypothesis on the direction of the outcome. The nature of missing data was analysed and addressed accordingly, using single imputation with unconditional means for data missing completely ad random,[30] as assessed through inspection and with the Little´s test in SPSS. A sensitivity analysis was performed for imputed outcomes. Statistical analyses of myograph experiments were performed using Prism (Graphpad Prism Software, San Diego, CA, USA). Other analyses were performed with SPSS statistical software package for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± SE, unless indicated otherwise.

Results

Blood Pressure and Heart Rate

Systolic as well as diastolic blood pressure greatly reduced with GPA compared to controls, by respectively 42.7 (5.5) and 35.6 (5.0) mm Hg at 4 weeks ($p<0.001$) (Figure 2 Panel A and B). (Baseline SBP/DBP 192.2(6.5)/145.4(5.8) with GPA vs 190.8(6.0)/140.9(6.0) mm Hg in controls; p=0.88; at 4 weeks this was SBP/DBP 164.6(3.2)/118.7(2.4) with GPA vs 207.3(4.5)/154.3(4.4) mm Hg in controls). Heart rate did not significantly change during the trial, and was respectively 442 (7) at 4 weeks in GPA vs. 454 (10) in controls (baseline 458 (12) with GPA vs 456 (12) in controls).

Figure 2. Effect of GPA Treatment on Blood Pressure and Clinical Parameters



These graphs show the large systolic (SBP) and diastolic blood pressure (DBP) decrements in beta-guanidino-propionic-acid-treated (black circles) vs. control spontaneously hypertensive rats (grey circles) from baseline to 4 weeks of intervention (n=8 in each group; Panel A and B). Heart rate (Panel C) was not significantly different throughout the trial. Food intake (Panel D), restricted till day 11, was significantly lower in the beta-guanidinopropionic acid treatment arm at day 3, increasing thereafter, with a concomitant reduction in body weight, which was not significantly different between treatment groups at week 4 (Panel E). Data are mean (SE). P values are respectively *<0.05; **<0.01; and ***<0.001.

General health, Food Intake, and Body Weight

Animals appeared healthy and displayed normal physical activity throughout the study, without adverse or unexpected effects. In the setting of reduction of food in both treatment arms in the first 10 days, GPA-fed and control animals consumed equal amounts of food during the trial except for the first 3 days, where GPA fed animals ate less than 15 grams/day, causing a brief drop in weight (Figure 2d, e). Thereafter, the food intake was similar in both treatment arms with no significant difference in body weight at week 4, when the blood pressure difference was at its peak. In line with this, systolic blood pressure at week 4 correlated significantly with the use of GPA (Pearson product-moment correlation coefficient (r) −0.9; 95% confidence interval, CI [−0.96 to −0.83] and

not with body weight. Hence, in multivariable regression analysis only GPA use not body weight was associated with blood pressure at week 4, constant SBP 267 [147 to 387]; beta of −43 mm Hg [CI −57 to −30 mm Hg] for GPA use, and −0.05 mmHg [−0.36 to 0.27] per gram body weight.

Heart Weight and Heart to Body Weight Ratio

At 4 weeks mean heart weight [GPA 987.5 (93.4) mg vs. control 937.5 (101.7) mg, p=0.723]; and mean heart weight-to-body weight ratio [GPA 2.9 (0.3) mg/g vs. control 2.7 (0.3), p=0.28] were not significantly different between intervention and control groups.

Mesenteric Artery and Aorta

From each rat in control and GPA groups, 3 to 4 (median 4) mesenteric were assessed. Mean vessel diameter (275.3; SE 7.3 in controls vs. 298.6; SE 11.7 with GPA) and maximum KPSS-induced contractile force (4.1; SE 0.4 mN/100 µm vessel diameter; vs. 4.4; SE 0.2 with GPA) did not differ significantly between groups. The study was not powered to assess differences in vasodilation, but we did expect a trend towards greater vasodilation after GPA than in controls, with potentially an increase in NO-dependent vasodilation. Mean vasodilation at 10^{-5} mmol/L methacholine in mesenteric artery after phenylephrine was 24% higher with GPA (52.0, vs. 41.7% in controls). After KPSS-NE, vascular relaxation was respectively 11.2% and 41.3% higher in GPA-treated rats for SNP and DNFB in mesenteric artery; and 20.4 and 18.1% higher in GPA treated rats for aorta metacholine and metacholine+L-NNA, but the comparison was statistically significant for DNFB and aorta metacholine only (v, Panel A to E).

Blood and Tissue Analyses

Plasma creatinine concentration was lower with GPA as expected (11.6 microgram/L with GPA vs. 24.1 in controls; p<0.001), while HDL cholesterol was significantly increased with GPA (Table 1). In accord with previous reports,[20] skeletal muscle ATP lowered with GPA, 1.05 (0.02) pmol/ µg protein vs. 1.35 (0.01) in controls, p<0.001); with an ATP to ADP ratio of 7.6 (SE 0.13) with GPA vs. 11.1 (SE 0.14) in controls, p<0.001). In the heart, changes were less pronounced as reported previously,[20] ATP respectively 1.6 (0.1) pmol/ µg protein with GPA vs. 1.9 (0.1) (p=0.03) in controls, but the difference in ATP to ADP ratio was not statistically significant, respectively 3.33 (0.4) for GPA and 3.42 (0.2) for controls, p=0.42.

In addition, we found evidence of higher renin mRNA in the kidney cortex with GPA, 1.7 (0.2) vs. 1.1 (0.2) in controls (p=0.03). Finally, tissue CK mRNA was reduced in skeletal muscle after GPA (Figure 3, Panel F and G).

Missing Data and Sensitivity Analysis

The follow up was complete. There were no drop outs, and all animals could be analysed for the primary outcome with an intent-to-treat analysis. For other outcomes, the most

Table 1. Comparative biochemical parameters of GPA-treated vs. control rats

	Baseline		Intervention	
	Control	GPA	Control	GPA
Creatine kinase	113.5 (18.9)	109.4 (14.2)	91.0 (53)	104.0 (10.5)
Creatinine	21.9 (0.9)	22.6 (0.9)	24.1 (0.7)	11.6 (0.7)*
Urea	6.5 (0.4)	6.9 (0.4)	6.9 (0.1)	7.4 (0.3)
Sodium	143.8 (0.4)	143.3 (0.5)	144.4 (1.2)	143.6(0.3)
Glucose	9.8 (0.4)	9.5 (0.3)	10.3 (0.3)	10.4 (0.3)
Total cholesterol	1.6 (0.1)	1.6 (0.0)	1.7 (0.0)	2.3 (0.2)*
HDL	1.3 (0.0)	1.3 (0.0)	1.3 (0.0)	1.9 (0.1)*
Non-HDL cholesterol	0.29 (0.03)	0.27 (0.03)	0.35 (0.02)	0.41 (0.05)
Triglycerides	0.8 (0.1)	0.9 (0.1)	0.7 (0.1)	0.8 (0.1)

Biochemical parameters in plasma of treated (n=8) and control (n=8) spontaneously hypertensive rats before and after 4 weeks of intervention with GPA. Data are mean (SE) in mmol/L, except for creatine kinase (IU/L) and creatinine (μmol/L). *p<0.01, for GPA vs. control

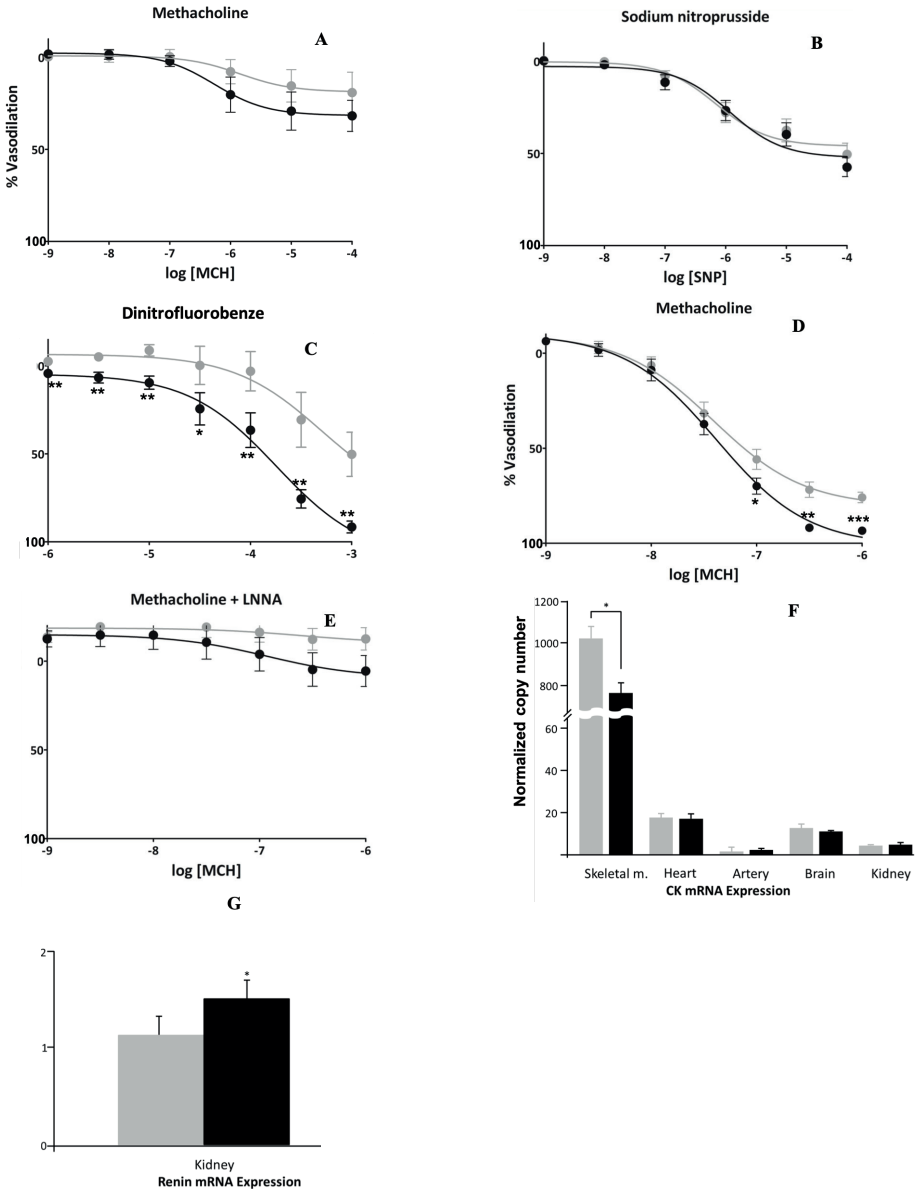
common cause for missing data was a lack of sufficient material for the planned analyses.

Missing data were baseline potassium, and plasma creatinine and ATP in skeletal muscle at 28 days (GPA, n=1); baseline plasma sodium and glucose at 28 days (both groups, n=1); and plasma sodium, potassium, and HDL after 4 weeks (control n=1). Missing data of the tissue studies are mentioned in Figure 3. Missing data where considered missing completely ad random by inspection and the Little´s test. The depicted outcomes are imputed data. As a sensitivity analysis, we reassessed all data without imputed values, and reanalysed data with non-parametric methods, without a change in the direction of these outcomes.

Discussion

To our knowledge, this is the first study with CK inhibition as a means to reduce blood pressure. Our data indicate very clearly that systemic CK inhibition reduces blood pressure effectively and safely in the SHR, an animal model of hypertension and high pre-existent CK activity in cardiac[7] and vascular tissue.[31] The results of this study are a new and important contribution to the body of evidence on the potential role of creatine kinase in hypertension. The antihypertensive effect of GPA supports the notion that CK is relevant for blood pressure.[1-9,13]

Figure 3. The Effect of GPA Treatment on Vascular Function and Tissue mRNA Expression



This figure depicts data on potential modes of action of beta-guanidinopropionic acid, vasodilation responses in isolated arteries (spline graphs; Panel A to E), and skeletal muscle creatine kinase and kidney renin mRNA (bar graphs; Panel F and G). Beta-guanidinopropionic acid, black squares and bars; controls, grey squares and bars). Vasodilation was induced after KPSS-NE by cumulative concentrations of methacholine (Panel A), sodium nitroprusside (Panel B), the irreversible creatine kinase inhibitor dinitrofluorobenzene (Panel C) in mesenteric artery (n=5 to 8 in each group); and in aorta after methacholine (Panel D) and after metacholine with N-omega-nitro-L-arginine (10^{-5}mol/L) (Panel E) (n=7 in each group). Data are expressed as percentage vasodilation of the maximum contractile response. Bars represent changes in relative expression of total cytoplasmic CK mRNA (n=7 to 8 in each group) (Panel F) and of kidney renin mRNA (n=4 to 8 in each group) (Panel G) after beta-guanidinopropionic acid treatment vs. controls. DNFB, dinitrofluorobenzene; MCH, methacholine; Skeletal m., skeletal muscle; SNP, sodium nitroprusside. *p<0.05; **p<0.01, ***p<0.001.

Regarding the mode of action, CK uses creatine to transfer energy-rich phosphoryl groups from intracellular generation to utilization sites as phosphocreatine.
The CK enzyme system is thought to promote hypertension through rapid regeneration of ATP from phosphocreatine near ATPases involved in particular in resistance artery contractility, but evidence indicates CK is also relevant for sodium retention.[1,8,14] In addition, the CK substrate creatine and nitric oxide (NO share a common precursor in L-arginine, and the high creatine synthesis that accompanies high CK activity is thought to reduce NO bioavailability.[2,32]

There is abundant evidence that GPA acts as a specific competitive inhibitor of creatine kinase, reducing the flux through the CK reaction, and thus of ATP-dependent responses.[1-3,5,6,13] However the effect of oral GPA on blood pressure was not addressed previously.[20] In this study, we found evidence of increased NO and CK-dependent vasodilation with GPA. Importantly, even a small decrease in the contractility of vascular smooth muscle could have a marked effect on arterial pressure, as blood flow and resistance in vivo are markedly affected by small changes in vessel caliber.[1-3] Thus, modest CK inhibition and vasodilation might have a large impact on blood pressure. In addition, creatine and NO share a common precursor in L-arginine, with creatine synthesis demanding a substantial part of the available L-arginine.[2] Hence, we speculate that competitive inhibition of GPA with creatine at AGAT[21] might also have resulted in greater bioavailability of L-arginine for NO synthesis, contributing to lower blood pressure.[2,3] In addition, we found kidney renin mRNA expression, reported to be compensatory increased by sodium depletion and diuretics,[33] to rise after GPA. This is in agreement with the proposed diuretic effect of CK, as the enzyme is known to be tightly bound near tubular basolateral Na^+/K^+-ATPase where it rapidly provides ATP for sodium retention throughout the kidney.[1,8,14]

Rats in the GPA treatment arm briefly lost weight in the first 3 days, compared to controls, rapidly regaining weight thereafter. At 4 weeks, when blood pressure in the GPA treated animals was - 43 mm Hg, there was no significant difference in body weight between treatment groups. Thus, weight reduction is unlikely to have contributed to the large reduction in blood pressure.

Rats treated with GPA showed a 46% increase in HDL-cholesterol. To our knowledge, this has not been assessed previously.[20] Rats are naturally deficient in cholesteryl ester transfer protein, with relatively high HDL and low non-HDL fractions.[34] HDL is well-known to have atheroprotective functions including a critical role in reverse cholesterol transport, as well as anti-inflammatory, antithrombotic, and antioxidant activity.[35] Theoretically, GPA might have increased HDL through several mechanisms including augmentation of the concentration of Apo A-I, mimicking of the functionality of Apo A-I, enhanced reverse cholesterol transport, or inhibition of endothelial lipase.[35] In line with the mitochondrial dependency of ABC transporter proteins,[36] we speculate that the stimulatory effect of GPA on mitochondrial function promotes reverse cholesterol transport. This is subject of our further studies.

Finally, the decreased skeletal muscle CK mRNA found in this study is in accord with lower skeletal muscle CK protein reported previously, due to a shift from high CK Type II fibers to low CK Type I fibers.[20] Since Type I fiber predominance displays a higher capillary density with greater NO generation and vasodilation resulting in lower peripheral resistance,[3] this might have contributed to the observed blood pressure lowering.
We carefully assessed the animals for potential side effects. In line with previous GPA studies and recent reports on knock-out mice deficient in creatine synthesis,[20,21,37] all experimental animals appeared healthy and normally active throughout the study without signs or symptoms of muscle, cardiac or brain dysfunction. High cardiac CK precedes hypertension in SHR, and increases thereafter,[7] and high CK and hypertension are both associated with ventricular hypertrophy.[38] However, to our knowledge there are no preexisting data on the effect of CK inhibition on the heart within the context of preexisting high tissue CK activity or hypertension. We expected that GPA might have reduced ventricular hypertrophy in our study, but despite the drop in blood pressure with GPA, heart rate, weight, and heart weight to body weight ratio did not significantly differ between the treatment and control group. These seemingly contradictory finding agrees with earlier reports on heart rate after blood pressure lowering in SHR, which showed an increase, a decrease, or no change after antihypertensive drugs,[26] and on the complex relationship between blood pressure and LVH in the SHR, where the level of blood pressure does not parallel the degree of cardiac hypertrophy.[39] Previous studies have confirmed an important role for an activated RAAS system with angiotensin II promoting cardiac hypertrophy in the SHR, whereas N-omega-nitro-L-arginine methyl ester attenuates this effect.[39] However, our study was not powered to assess these differences, and dedicated studies are needed to further assess these outcomes in hypertension.

The main strength of this study is that we explore, to our knowledge for the first time, the effect of CK inhibition on blood pressure. The SHR, an animal model of hypertension, is also reported to express high CK activity, [2,7] Therefore, our data . also provide new insights into how a high CK system might respond to CK inhibition, and thus help understand the (patho)physiology of high CK activity. We are not aware of previous studies on this topic. Although our sample size was relatively small, it was adequate to assess the primary outcome with sufficient power. We used stringent methodological and procedural measures to ensure the welfare and limited use of animals according to the ARRIVE guidelines[15] and to correctly assess the primary outcome, including randomization, concealment of treatment allocation, and standardization the tail cuff blood pressure measurements. Furthermore, we assessed the potential mode of action in non-primary outcomes including vasodilation and renin expression.

The presented data indicate that intracellular creatine reduction and reversible competitive creatine kinase inhibition with GPA may reduce systemic arterial blood pressure in hypertension.This experimental evidence adds to the data from animal and human studies indicating that creatine kinase is a main determinant of blood pressure and of resistance artery contractility, as well as of failure of antihypertensive therapy in the general population.[1-7,13]

Blood pressure reduction in hypertensive patients may be challenging despite the availability of several classes of antihypertensive drugs, and a substantial proportion of treated hypertensive patients does not achieve blood pressure control despite adequate treatment.[13,40-43] Risk factors for poor hypertension control include obesity, age, African ancestry, the presence of diabetes or end organ damage; but non-adherence of the patient, the white-coat effect, therapeutic inertia of the physician, dietary factors, or the concomitant use of blood pressure increasing drugs may also contribute. However, a subgroup of patients with uncomplicated, primary hypertension remains uncontrolled despite adequate use of antihypertensive drugs, and antihypertensive acting via new mechanisms might aid in achieving better control in these patients.[41-43] beta-guanidinopropionic acid is synthesized by the mammalian kidney, has no xenobiotic metabolism, and has been sold online to be used as a food supplement by athletes.[44] Therefore we recently conducted a proper first in human study with low dose GPA, which was uneventfull (Karamat et al., in preparation). Thus GPA is a promising substance to further evaluate the potential role of creatine kinase in the generation of high blood pressure.

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Conflicts of interest

There are no conflicts of interest.

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Chapter 6

The acute effect of beta-guanidinopropionic acid versus creatine or placebo in healthy men (ABC Trial): study protocol for a randomized controlled trial

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Trials. 2015; 16:56

Abstract

Background: Despite adequate treatment, up to 30% of treated antihypertensive patients with primary, uncomplicated hypertension remain uncontrolled. We proposed that high intracellular activity of the ATP regenerating enzyme creatine kinase increases pressor responses and hypertension risk. In line with this, we found that that plasma activity after rest, a surrogate measure of tissue activity, is the main predictor of blood pressure levels and failure of antihypertensive therapy in the general population. In addition, the creatine analogue and competitive oral creatine kinase inhibitor beta-guanidinopropionic acid effectively and safely reduced blood pressure in the spontaneously hypertensive rat. However, to our knowledge there are no human data on the safety of oral supplementation with this substance. Therefore, we will assess the tolerability of beta-guanidinopropionic acid in men, compared to creatine and placebo.

Methods/Design: This is a randomised, active and placebo controlled, triple blind, double dummy, single centre clinical intervention trial in 24 healthy male volunteers, 18 to 50 years old, recruited in the Netherlands. The intervention consists of one week of daily oral administration of beta-guanidinopropionic acid 100 mg, creatine 5 gram, or placebo. The primary outcome is the tolerability of beta-guanidinopropionic acid as a descriptive measure, in an intent to treat analysis. Other outcomes include the placebo adjusted differences with baseline in biochemical and haemodynamic parameters, including plasma markers of muscle tissue damage, urine sodium excretion, resting sitting systolic and diastolic brachial blood pressure, supine systolic and diastolic central blood pressure, pulse wave velocity and augmentation index, heart rate, cardiac contractility, cardiac output, and total peripheral resistance.

Results: There is an unfulfilled need for new conservative options to treat resistant hypertension. This study will provide first-in-men data on creatine kinase inhibition as a potential new class of antihypertensive drugs.

Discussion: There is an unfulfilled need for new conservative options to treat resistant hypertension. This study will provide first-in-men data on creatine kinase inhibition as a potential new class of antihypertensive drugs.

Trial registration: The Netherlands National Trial Register Trialregister.nl (identifier NTR 4444) , registered 9March 2014.

Keywords: creatine kinase, beta-guanidinopropionic acid, creatine, tolerability, blood pressure

Background

Blood pressure reduction may be challenging despite the availability of several classes of antihypertensive drugs [1-5]. A substantial proportion of treated hypertensive patients, up to 30% or more does not achieve blood pressure control. Risk factors for poor control include obesity, age, African ancestry, the presence of diabetes or end organ damage; but non-adherence of the patient, the white-coat effect, therapeutic inertia of the physician, or the concomitant use of blood pressure increasing drugs may also contribute. However, a subgroup of patients with uncomplicated, primary hypertension remains who are uncontrolled despite adequate use of antihypertensive drugs [1-5]. The underlying pathophysiology in these patients is thought to be refractory to currently available drugs, causing early heart disease, stroke, and early mortality. Hence, the current scientific challenge is to develop new conservative options to lower blood pressure [1-5].

We showed in a random, multi-ethnic population sample that plasma CK activity after rest, a surrogate measure of tissue CK, is the main predictor of blood pressure, with a crude increase in blood pressure of 14 mm Hg systolic and 8 mm Hg diastolic per log CK increase[6]. Although plasma and tissue CK activity were found to be higher in men, subjects of African ancestry, and obese patients [6,7], the association was independent of sex, body mass index (BMI), or ethnicity. Therefore, we proposed that high tissue CK might increasepressor responses [6].

Cytosolic CK is tightly bound in the immediate proximity of ATP-utilizing enzymes such as Na^+/K^+-ATPase, Ca^{2+}-ATPase, and myosin ATPase. Here, ATP synthesized by CK is preferentially used to fuel highly energy-demanding processes such as sodium retention, cardiovascular contractility, as well as remodeling of arteries, promoting hypertension [6,8]. Importantly, in accord with a causal relationship, high tissue CK preceded hypertension in animal models [9,10], as was found with high plasma CK in humans [11], and inhibition of intracellular CK substantially inhibited human vascular contractility in vitro [12]. Furthermore, vascular CK gene expression was strongly associated with clinical blood pressure in humans[13], and high plasma CK was found to be the main predictor of failure of antihypertensive therapy in the general population [5,14]. Finally, we recently showed in a randomized control trial of 16-week-old male spontaneously hypertensive rats versus controls (n = 16), that oral CK inhibition with the competitive CK inhibitor beta-guanidinopropionic acid (GPA) 3%, added to rat chow over 4 weeks, safely reduced blood pressure. With a systolicand diastolic baseline blood pressure of respectively 191.5 (SE 4.3) and 143.1 (SE 4.1) mm Hg, GPA significantly reduced blood pressure compared to controls by 42.7 (5.5) systolic and 35.3 (4.8) mm Hg diastolic ($P < 0.001$), respectively [15]. To our knowledge, there are no human data on the safety and effects of this potential new antihypertensive agent; we will assess the tolerability of GPA in healthy volunteers.

Methods/Design

Test product

GPA

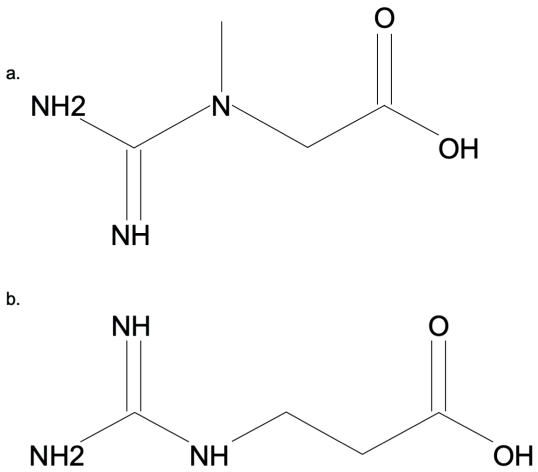
GPA or N-(aminoiminomethyl)-beta-alanine; ($C_4H_9N_3O_2$), is a structural isomer of creatine (Figure 1) [16]. GPA is generated in vivo via transamidination of β-alanine (Figure 2) [17-19]. The physiological concentration in human plasma is reported to range from trace amounts to 1.40 µmol/L [20,21]. Clearance is probably renal, akin to creatine, creatinine, and other guanidino compounds [17,19-21]. Despite the lack of human data on efficacy and side effects, GPA is available as a foodsupplement,usually in doses of 500 mg, and is used by sportspersons to induce endurance capacity and promote weight loss [16]. GPA acts as a competitive inhibitor of cellular creatine uptake, and attenuates the flux through the cytoplasmic creatine kinase reaction [15,16,18]. CK catalyses the rapid and reversible transfer of a phosphoryl group from creatine phosphate to ADP, thereby forming creatine and ATP:

ADP + creatine phosphate <=> ATP + creatine.

The flux through the CK reaction is linearly correlated to the concentration of creatine[22]. Nuclear genes encode four CK subunits or monomers: cytoplasmic muscle (CKM), cytoplasmic brain (CKB), ubiquitous mitochondrial (CKMT1), and sarcomeric mitochondrial (CKMT2). The enzymatic functional form can be either a homodimer (BB and MM), a heterodimer (MB), or an octamer (mitochondrial monomers), thus creating five isoenzymes. The three dimeric cytoplasmic CKMM, CKMB, and CKBB isoenzymes are predominantly expressed in striated skeletal and heart muscle (MM), heart muscle (MB); and brain and smooth muscle (BB). The two octameric mitochondrial CK isoenzymes are expressed in striated muscle and other tissue [13]. The cytoplasmic isoenzymes appear in plasma of healthy subjects, due to what is thought to be a nontraumatic proportional release from tissue entering the bloodstream through the lymphatic system [6]. Hence, plasma CK is mainly the CKMM isoenzyme.

GPA is thought to inhibit cytoplasmic, but not mitochondrial CK [15,16,18]. Although GPA is phosphorylated by cytoplasmic CK, both GPA and phosphorylated GPA are ‘inefficient substrates’ for the CK reaction: in vitro Vmax values are <1% of the Vmax values of creatine and phosphocreatine [16,18,23]. Therefore, GPA may modulate the energy status of tissues, and we speculated that this creatine analog may reduce blood pressure. In animal studies, supplemental GPA (1 to 3%) in the diet led to skeletal muscle changes similar to the adaptations of endurance training [16]. In the unstressed heart, left ventricular systolic pressure, cardiac output, and rate of tension development were unchanged with GPA. During high workload, studies showed unchanged or reduced peak left ventricular developed pressure and cardiac output. However, blood pressure and peripheral hemodynamic parameters were not an outcome in these studies [16]. We recently showed that feeding 16-week-old spontaneously hypertensive rats a diet containing 3% GPA reduced blood pressure [15]. Importantly, the animals appeared

FIGURE 1. Structural analogy between creatine and beta-guanidinopropionic acid



Legend Creatine (a) and beta-guanidinopropionic acid (b) have an identical molecular formula ($C_4H_9N_3O_2$), but creatine is methylated on its tertiary nitrogen, whilst in beta-guanidinopropionic acid the methyl group is positioned in the carbon chain [16].

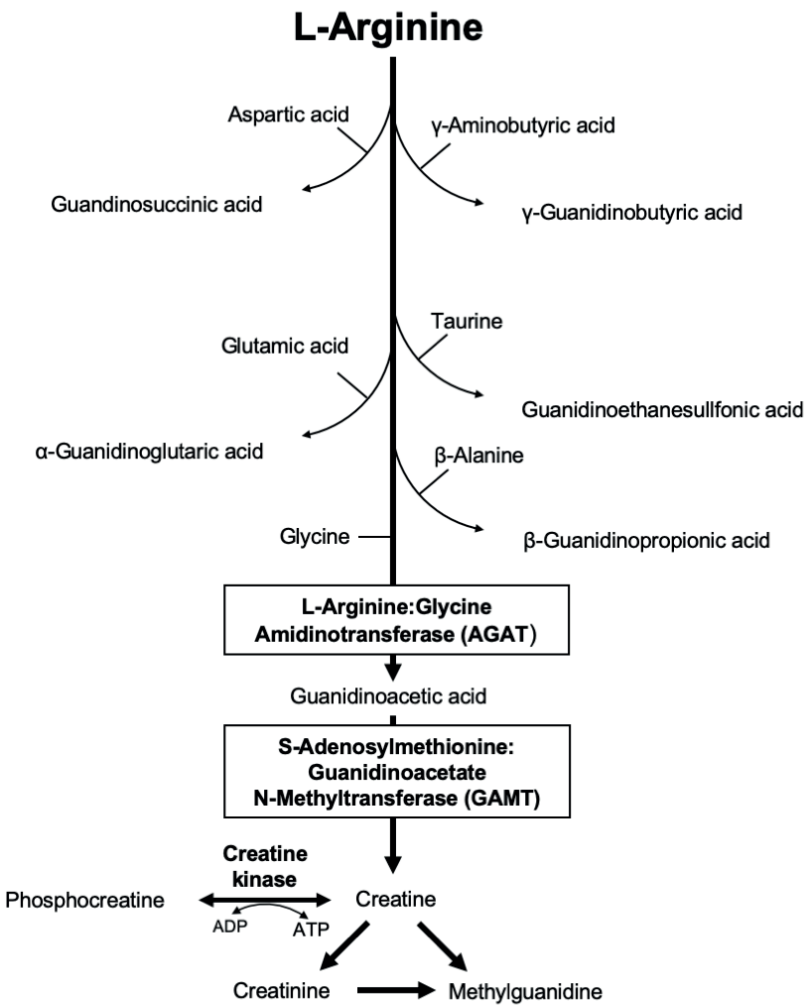
healthy after GPA [15,16], and the blood pressure-lowering effect was reversible after withdrawal of the analog [15].

Manufacturing and testing

In accord with the definition for food supplements in the legislation of the European Union[24], we consider GPA as well as creatine to be food supplements, because both are naturally occurring amino-acids. GPA is a white crystalline tasteless powder, soluble in water. GPA powder is ordered at Sequoia (Sequoia Research Products, Oxford, UK). There are no reports or bans on this product or the company to our knowledge, presented on the FDA website using the FDA search engine, or online with search engine Google, as of 20 February 2014. GPA, creatine, and identical placebo capsules will be manufactured by the Pharmacy & Pharmacology Department of the Slotervaart Hospital, Amsterdam, The Netherlands. This department is GMP certificated (ISO 9001:2001). Product dossiers for GPA and creatine were written and have received formal ethical approval by the AMC Amsterdam Medical Ethics Review Committee (MERC). GPA is marketed for human use in the U.S. and Australia, but not in European countries. According to the legal guidelines of the European Union, criteria of international organs, generally accepted criteria, or national criteria are approved when a supplement is not listed in the legislation of the European Union.

Following the U.S. FDA guidelines [25], we first qualified the supplier by establishing the reliability of the supplier, with the methods mentioned above. Next, the substance was

FIGURE 2. Pathways of guanidino compound synthesis.



Legend Guanidino compounds such as beta-guanidinopropionic acid, creatine, guanidinoacetic acid, gamma-guanidinobutyric acid, and guanidinosuccinic acid, are reported to be synthesized via transamidination of the amidino group from arginine as the major pathway, or through the urea cycle. Creatine biosynthesis involves two sequential steps catalyzed by L-arginine:glycine amidinotransferase (AGAT), and S-adenosylmethionine: guanidinoacetate N-methyltransferase (GAMT) After [17-19].

tested for purity and for cyanide compounds. Cyanide was not expected to be quantifiable [26]. However, the cyano-group in cyanamide, one of the compounds used in the formation of GPA, provides a possible source of cyanide. We established in our certified tests a purity of more than 99% (detection limit) and a cyanide level lower than 1 p.p.m. (detection limit). Cyanide occurs in many food items, with high concentrations in cassava roots, almonds, and apricot kernels, up to 7,000 mg/kg (7 parts per thousand) [27].
In Europe, Annex II of Directive 88/388/EEC on flavorings sets the following maximum levels for hydrocyanic acid in foodstuffs and beverages: 1 mg/kg in food or beverages, with the exception of 50 mg/kg in nougat, marzipan or similar products, and 5 mg/kg in canned stone fruit [28]. With 100 mg GPA containing <1 p.p.m. cyanide, the contribution to the daily intake will be <0.1 microgram/day.

Storage and distribution

GPA and creatine capsules will be stored at room temperature. The participants will receive the test products at the hospital and will be instructed to store the capsules at room temperature at home.
The test products will be labelled with a study number, with the Pharmacy holding the key to the content until the end of the data collection.

Dose calculation

For GPA, we used the FDA guidance on Estimating the Maximum Safe Starting Dose in Initial Clinical Trials in Adult Healthy Volunteers[29]. This guidance outlines a process for deriving the maximum recommended starting dose for first-in-human clinical trials in adult healthy volunteers, and recommends a standardized process by which the maximum recommended starting dose can be selected. The purpose of this process is to ensure the safety of the human volunteers[29]. Toxicity should be avoided at the initial clinical dose. However, doses should be chosen that allow reasonably rapid attainment of the phase 1 trial objectives[29].
The major elements of this process are

1. Determination of the no observed adverse effect levels (NOAELs) in the tested animal species
2. Conversion of NOAELs to human equivalent doses (HED)
3. Application of a safety factor

1. No observed adverse effect level (NOAEL) determination
In animal studies, GPA was administered through the diet in concentrations of 1% or more over 8 weeks without apparent adverse effects [16]. In animals weighing 200 grams eating 20 grams per day, we calculated a 'no observed adverse effect level' of 1,000 mg/kg/day. Furthermore, in a patent application, Meglasson et al. recommended a human dose of 1 to 500 mg/kg/day based on his research in mice and rhesus monkeys [30]. In this paper, rhesus monkeys weighing 9 kg were treated with oral GPA 48 mg/kg/day (432 mg per monkey per day) over 2 weeks without apparent adverse events.

2. Conversion of the no observed adverse effect level (NOAEL) to human equivalent dose (HED)

We converted the oral NOAELs in rats and monkeys (resp. 1,000 mg/kg/day and 48 mg/kg/day) to oral HEDs based on an algorithm proposed by the FDA based on body surface area[29]. This algorithm proposes a conversion factor from rat to human of 0.16 times the rat dose; and of monkey to men of 0.32 the monkey dose (in mg/kg/day; for a man of 60 kg) resulting in HEDs of resp. 160 mg/kg/day and 15 mg/kg/day for a man of 60 kg.

3. Application of a safety factor

A safety factor should be applied to the HED to increase assurance that the first dose in humans will not cause adverse effects. The use of the safety factor is based on the possibility that humans may be more sensitive to the toxic effects of a substance than predicted by the animal models, that bioavailability may vary across species, and that the models tested do not evaluate all possible human toxicities, or cannot be expressed by animals or easily measured, such as headache or nausea. We conservatively chose 15 mg/kg/day oral dose for our final calculations of the human dose, because this is the lowest dose, and because of the closer allometric relationship between monkey and man [29]. FDA advises a safety factor of at least 10. Based on an average weight of a male volunteer of 75 kg, we calculated a starting oral dose for this phase 1 study of 75*1.5 mg/day = 112.5 mg/day; we will use 100 mg/day.

Creatine

Creatine, which has an identical molecular formula as GPA, was chosen to simultaneously assess the effect of the synergist on peripheral hemodynamics. The average daily rate of creatine synthesis in healthy omnivorous males is estimated to be 1.3 g [31]. We will use 5 g as recommended in studies on creatine supplementation. No side effects are apparent at this dose [31,32].

Study Design

We wrote the protocol using the SPIRIT '(Standard Protocol Items: Recommendations for Interventional Trials)' recommendations and the 'Template for Intervention Description and Replication (TIDieR) checklist' [33,34]. The ABC trial is a randomized, placebo and active controlled, double-dummy, participant, intervention provider, and outcome assessor (triple) blinded, parallel group, single center (Academic Hospital of the University of Amsterdam), exploratory trial, with three arms: a primary outcome of tolerability of GPA, in comparison with creatine and placebo. Randomization will be performed by an independent party, the Clinical Pharmacy Unit of the Academic Hospital of the University of Amsterdam, using a computer-generated, nonadaptive, and restricted randomization scheme and a 1:1:1 allocation ratio. The Pharmacy will generate the random allocation sequence. All participants who give consent for participation and who fulfill the inclusion criteria will be randomized to receive GPA 100 mg and creatine placebo matching active creatine 5 gram; creatine 5 gram and GPA placebo matching GPA 100 mg; or double dummy placebo over 1 week. The participant will receive the blinded, randomized trial supplements from the pharmacy. Allocation

concealment will be ensured, as the pharmacy will store the allocation list and not release the randomization code until all outcome measures have been assessed and the data bank has been closed. Thus, randomization will be conducted without any influence of the investigators, outcome assessors, or participant characteristics. After assignment to interventions, trial participants, trial staff, and the outcome assessor will remain blinded to whether the participant was given a placebo or a supplement until after all outcome data have been assessed. Data analysis will be performed unblinded. We prespecified the use of accumulating data to decide whether to stop the trial early, in case of serious or unexpected side effects. Independent monitoring visits will take place before the start of the trial, within 1 month after initiation or after inclusion 5th subject; within 2 months after initiation or after inclusion 15th subject; after last patient last visit; and after the data have been entered into the database. Data entry will be verified by two independent researchers. Budget administration is by an independent organization (AMC Research BV, Amsterdam, the Netherlands).

Objectives

The primary objective is to assess the tolerability of one week of 100 mg oral GPA daily, as compared to placebo. Secondary objectives include the comparison of tolerability with creatine, and the effect of one week of oral GPA on hemodynamic parameters, including peripheral and central blood pressure, and cardiac contractility as compared to creatine and placebo. The tertiary objective is to assess the effect of one week of oral administration of GPA on biochemical parameters, including ADP-induced platelet aggregation [35], compared to creatine and placebo.

Eligibility

We will include healthy men aged 18 to 50 years, with a normal, nonobese body mass (BMI 18.5 to 29.9 kg/m2). Exclusion criteria include high blood pressure or the use of antihypertensive drugs at baseline, (history of) cardiovascular disease including TIA and stroke; the use of plasma CK-increasing drugs including statins; use of acetylsalicylic acid or nonsteroidal anti-inflammatory drugs in the two weeks prior to the first visit; neuromuscular or endocrine disorders; vasculitis; HIV infection; infectious hepatitis; personal or family history of bleeding disorders; sickle cell anemia or other hereditary anemia; smoking; current use or use within two months prior to start of the trial of beta-guanidinopropionic acid or creatine; and abnormalities in glucose, lipid spectrum, thyroid, kidney, or liver biochemistry parameters in the plasma. To stabilize and standardize plasma CK activity during the trial, participants are instructed not to perform exercise three days prior to the baseline visit or during the intervention in the first week [6].

Statistical analysis

Study outcomes

The primary outcome is the tolerability of GPA after oral administration in healthy male volunteers versus placebo as a descriptive measure, in an intent-to-treat analysis.
Other outcomes are to compare the tolerability of GPA with creatine, and differences in hemodynamic and biochemical parameters between treatment arms.

Sample size calculation

This is a first-in-man study with GPA, with allometric data available from other species. According to the EMEA guidelines [36], we will include 8 subjects in each arm, to assess tolerability of GPA versus placebo and creatine during one week.

Recruitment Strategy

We will utilize two primary resources for identifying and recruiting potential subjects, advertising and identification in our Healthy Volunteer Research Database. The advertisement has prior approval of the MERC. A dedicated trial staff member will respond to inquiries about participation in the trial on the same day, using a participant information letter approved by the MERC. Screening will continue until the target population is achieved (24 subjects). The enrolment period is planned to extend over 9 months, till December 2014.

Clinical Investigation

The participants will be instructed to come to the hospital in the morning after an overnight fast for all visits during the intervention, which is the first week of the trial (Visit 1 to 5).

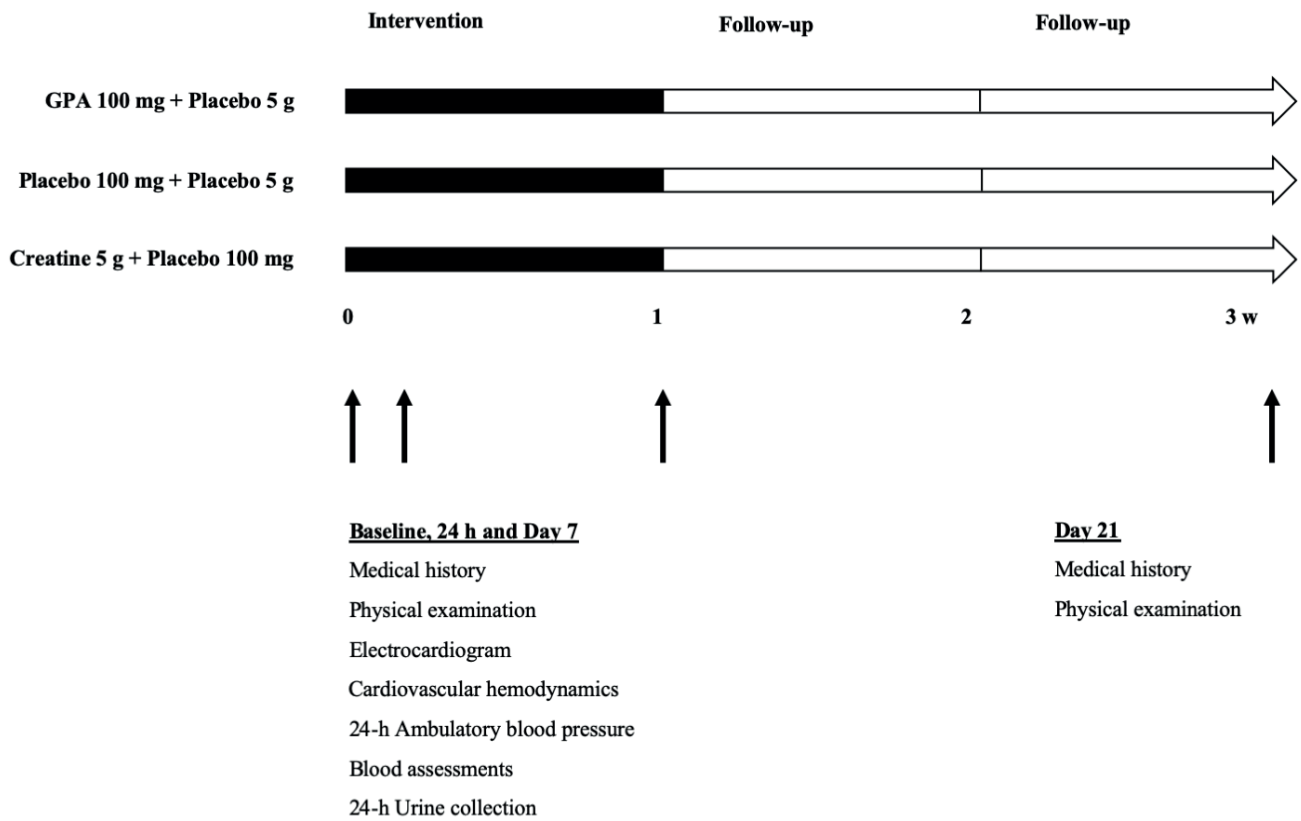
Intervention

The intervention will be provided under supervision of a medical doctor. The duration of the intervention will be 7 days. To ensure intervention adherence, trial supplements are ingested by the volunteer in the presence of the trial staff during the hospital visits. In addition we will use pill counts for the supplements taken at home.

Time line clinical studies

The time line is depicted in Figure 3. In brief, clinical studies will be performed at baseline, and 1 day and 7 days after the trial supplements are used. The final assessment of tolerability is at day 21.

FIGURE 3. Trial time line



Legend The duration of the intervention is 7 days. The trial supplements start at Day 1, after baseline measurements, inclusion and randomisation at Day 0 (baseline). After 24-h and 7 days of trial supplements, baseline measurements will be repeated, with the last visit at Day 21, to assess side effects.

Questionnaire

The participants will receive a questionnaire to assess tolerability at home during the week of intervention and in the 2 weeks after the intervention. The questionnaire encompasses the perceived side effects of the trial supplements, using check boxes and free text space. The questionnaire was piloted with healthcare professionals, and healthy volunteers.

Electrocardiography and Haemodynamics

On Day 0 (baseline), 1 (in the first hour after intake trial supplements), 2 (after 1 day of trial supplements), and 8 (after 7 days of trial supplements) of the intervention period, we will measure sitting brachial systolic and diastolic blood pressure Omron M4 oscillometric device: Omron Healthcare Europe BV, (Hoofddorp, the Netherlands) after 5 minutes of rest with an adjusted cuff size on the left arm, at heart level. In addition, we will perform electrocardiography (MAC 5000 Resting ECG System; GE Healthcare; Boston, MA) and ambulatory 24-hour blood pressure monitoring (Spacelabs 90217 Ambulatory Blood Pressure Monitor, Spacelabs Inc. Redmond, WA, USA). Furthermore, at baseline, day 2, and day 8, we will estimate central blood pressure, pulse wave velocity, and the augmentation index with a Arteriograph (Tensiomed Kft, Budapest, Hungary); and heart rate, cardiac contractility, cardiac output and total peripheral resistance using a Nexfin BMEYE, (Amsterdam, the Netherlands) blood-pressure monitor for continuous non-invasive finger arterial blood pressure measurement.

Laboratory studies

All laboratory studies are after an overnight fast. At baseline, we will assess plasma GPA, resting plasma CK (after 3 days without heavy exercise), glucose, insulin, lipid profile, creatine, creatinine, liver enzymes (ASAT, ALAT, gamma GT), LDH, cardiac troponin, myoglobin, TSH (to exclude subclinical hypothyroidism associated with high plasma CK), sodium, potassium, platelet count, coagulation tests (aPTT, PT), and ADP-induced platelet aggregation (area under curve at final concentration ADP 0.1, 0.2, 0.5, 1 and 2 µmol/L). Furthermore, in collected 24-h urine we will assess GPA, creatine, creatinine, urea, sodium, and potassium. Tests will be repeated after 1 day, and after 7 days of trial supplements, with the exception of TSH, aPTT, and PT. ADP-induced platelet aggregation will repeated at Day 8 only.

Concomitant care and interventions

There is no relevant concomitant care and no other interventions are permitted during this trial of healthy volunteers.

Safety

The investigator will inform the subjects and the MERC if any event occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater

than was foreseen in the research proposal. The study will be suspended pending further review by the MERC, except insofar as suspension would jeopardise the subjects' health. The investigator will take care that all subjects are kept informed.

Adverse and serious adverse events

We do not expect any adverse effects from this low dose study. There are no FDA or other reports in formal or informal sources on the side effects of ingestion of this dose of GPA or creatine in animals or humans. Therefore, a 1-day first in men study we had proposed, with hourly observation and physical and laboratory examination of the subjects was deemed unnecessary by the MERC. Subjects with hypertension, a history of cardiovascular, liver, or kidney disease, or with laboratory abnormalities at baseline will be excluded. Adverse events reported by the included subject spontaneously or through the questionnaire, or observed by the trial staff or health care worker, will be recorded and judged by the study group and the independent physician. The trial staff will convert reported symptoms to a standard lexicon, the Common Terminology Criteria for Adverse Events (CTCAE) [37], to facilitate international scientific reporting. Adverse effects will be classified based on the FDA guideline [29], as overt toxicity (e.g., clinical signs, macro- and microscopic lesions); surrogate markers of toxicity (e.g., plasma liver enzyme levels); or other adverse effects. The ADR probability scale [38] will be used to assess the causal relationship between trial supplement use and any reported adverse event. Adverse events will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures.

Retention and withdrawal of individual subjects

We will actively monitor retention, and once enrolled, we will make every reasonable effort to follow the participant for the entire study period. In the trial design, we put great effort into limiting the participant's burden related to visits and procedures, including the calculation of an appropriate NOAEL and HED, and the predominant use of non-invasive assessments. Participants will receive a financial compensation within the MERC guidelines. However, subjects can leave the study at any time for any reason without any consequences. Participants will be given the option to be followed up on certain outcome measures only, if this would lead to retention. Non-adherence will in itself not be a reason to exclude the participant. We will collect the reasons for non-adherence and non-retention where possible. In addition, the investigator can decide to withdraw a subject from the study for any medical reasons. Upon withdrawal, subjects will be replaced.

Emergency unblinding

To ensure the overall quality of the trial, code breaks will occur only when knowledge of the actual supplement given is absolutely essential for further management of the participant. This will be decided by the independent physician. Premature termination of the study If despite our expectations, any serious side effect is observed in the

volunteers, the study may be stopped prematurely.

Quality Control

We will ensure that quality controls will be executed throughout the conduct of the study, with regard to participant selection, data collection, data processing and reporting. Additionally, trial staff who collects the data will be well-trained according to standard operating procedures, in the study requirements, use of the questionnaire, counselling for adherence, standardized measurement of height, weight, brachial blood pressure, electrocardiography and non-invasive hemodynamics, as well as for requirements for laboratory specimen collection including morning urine samples. On every day of the data collection, we will monitor and ascertain the performance of our measurement devices. Our database was designed to allow checks on the completeness of the entered data and basic data checks and we used independent double data-entry followed by matching and checking for data-entry errors. Data cleaning will be performed according to expert consensus. Finally, we will check internal and external consistency of the analyzed data before writing reports.

Ethical considerations

The study will be conducted according to the principles of the Declaration of Helsinki (Adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, and amended by the 59th WMA General Assembly, Seoul, October 2008), and in accordance with the Dutch Medical Research Involving Human Subjects Act (WMO). Prior to undertaking any study related procedures, each participant will receive a verbal and written explanation of study aims, methods, and potential side effects. The participants will provide written informed consent. The full study protocol is approved by the AMC Amsterdam Medical Ethics Review Committee on 25 November 2013 (MERC reference number 38368.018.12). All study-related information will be stored securely at the study site and participants' personal study information will not be released without his written permission.

Handling and storage of data and documents

Handling of personal data will comply with the Dutch Personal Data Protection Act. Data will be entered anonymously in a database designed for the study, with a code of which the key will be held by the clinical project leader (LMB). The original study forms, extracted data, and biological samples will be kept at the hospital for 15 years, with access restricted to the trial staff.

Discussion

Hypertension is still the main risk factor for premature death [1]. Despite the ample availability of antihypertensive drugs and the adequate use whereof, it is estimated that around 10 to 30% of the hypertensive patients are not controlled with currently available

drug regimens. Currently, there is an unfulfilled need for new conservative options to treat resistant hypertension [1-5].

This study is based on incremental data indicating that the ATP regenerating enzyme creatine kinase enhances the energy demanding processes involved in hypertension, including vascular contractility and salt retention, and that the creatine analog and competitive CK inhibitor GPA reduces blood pressure in animal studies [5,6,8-16]. Hence, this is a first-in-men study of what might become a new class of antihypertensive drugs.

In this study, we will collect data with close adherence to the US FDA and European guidelines. We expect no difference in reported adverse effects between GPA, creatine, and placebo. This study will increase the knowledge on the effect of moderate reversible cytoplasmic creatine kinase inhibition on the human cardiovascular system and provide data on tolerability and hemodynamic parameters. Beta-guanidinopropionic acid doses are low, aimed at preventing

Trial status

The trial is currently recruiting participants.

List of abbreviations

AGAT: L-arginine:glycine amidinotransferase; BMI: body mass index; CK: creatine kinase; CKB: cytoplasmic brain CK; CKM: cytoplasmic muscle CK; CKMT1: ubiquitous mitochondrial CK; CKMT2: sarcomeric mitochondrial CK; CTCAE: Common Terminology Criteria for Adverse
Events; EMEA: European Medicines Agency; GAMT: S-adenosylmethionine: guanidinoacetate N-methyltransferase; GPA: beta-guanidinopropionic acid; HED: human equivalent doses; MERC: Medical Ethics Review Committee; NOAEL: no observed adverse effect level; SPIRIT: Standard
Protocol Items: Recommendations for Interventional Trials; TIDieR: Template for Intervention Description and Replication.

Competing interests

L.B. is an inventor on NL patent WO/2012/138226 (filed).

Authors' contributions

FK participated in the design of the study, drafted the manuscript, and will conduct the clinical studies and the primary statistical analysis. **DH** participated in the design of the study, drafted the manuscript, and will conduct the clinical studies and the primary statistical analysis. **YH** drafted the manuscript, and will conduct the clinical studies and the primary statistical analysis. **LW** participated in the design of the study, and will conduct the clinical studies. **IO** initiated the study design, drafted the manuscript, and will supervise the clinical studies. **GM** participated in the design of the study and will supervise the clinical studies. **JC** participated in the design of the study. **LB**, the grant holder, conceived the study, initiated the study design, provided statistical expertise in the clinical trial design, will supervise the clinical studies, and conduct and supervise the statistical analyses. All authors contributed to the writing of the study protocol, and read and approved the final manuscript.

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Chapter 7

The acute effect of beta-guanidinopropionic acid versus creatine or placebo in healthy men (ABC-Trial): a randomized controlled first-in-human trial

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Abstract

Aim: Increasing evidence indicates that the ATP-generating enzyme creatine kinase (CK) is involved in hypertension. CK rapidly regenerates ATP from creatine phosphate and ADP. Recently, we showed that beta-guanidinopropionic acid (GPA), a kidney-synthesized creatine analogue and competitive CK inhibitor, reduced blood pressure in spontaneously hypertensive rats. To further develop the substance as a potential blood pressure-lowering agent, we assessed the tolerability of a sub-therapeutic GPA dose in healthy men.

Methods: In this active and placebo-controlled, triple-blind, single-center trial, we recruited 24 healthy men (18 to 50 years old, BMI 18.5 to 29.9 kg/m2) in the Netherlands. Participants were randomized (1:1:1) to one week daily oral administration of GPA 100 mg, creatine 5 gram, or matching placebo. The primary outcome was the tolerability of GPA, in an intent-to-treat analysis.

Results: Twenty four randomized participants received the allocated intervention and 23 completed the study. One participant in the placebo arm dropped out for personal reasons. GPA was well tolerated, without serious or severe adverse events. No abnormalities were reported with GPA use in clinical safety parameters, including physical examination, laboratory studies, or 12-Lead ECG. At day 8, mean plasma GPA was 213.88 (SE 0.07) in the GPA arm vs. 32.75 (0.00) nmol/L in the placebo arm, a mean difference of 181.13 (95% CI 26.53 to 335.72).

Conclusion: In this first-in-human trial, low-dose GPA was safe and well-tolerated when used during 1 week in healthy men. Subsequent studies should focus on human pharmacokinetic and pharmacodynamic assessments with different doses.

Clinical trial registration number: The Netherlands National Trial Register (NTR) number 4444, registered March 9, 2014

What is known about this subject
- Plasma activity of the creatine kinase (CK), the energy enzyme that rapidly regenerates ATP from phosphocreatine, is associated with blood pressure in the general population; a crude increase of 14 mm Hg in systolic pressure per log CK increase
- High CK activity precedes the development of hypertension in animal and human studies
- CK inhibition with beta-guanidinopropionic acid (GPA), a kidney-synthesized creatine analogue, lowers blood pressure in an animal hypertension without apparent side effects

What this study adds
- This first-in-human study in healthy men, who orally ingested a daily dose of 100 mg GPA during one week raised no safety or tolerability concerns, including no adverse effects reported and no significant differences detected compared to baseline or placebo in physical examination, biochemistry, or cardiovascular function including blood pressure, cardiac contractility, and QT interval.

Introduction

There is increasing evidence that creatine kinase (CK, EC 2.7.3.2) is intimately involved in the generation of blood pressure.[1-6] CK catalyses the rapid and reversible transfer of a phosphoryl group from creatine phosphate to ADP, thereby forming creatine and ATP:[1-6]

$$\text{Creatine phosphate} + \text{MgADP} \leftrightarrow \text{Creatine} + \text{MgATP}$$

Cytosolic CK is tightly bound in the immediate proximity of ATP-utilizing enzymes such as Na^+/K^+-ATPase, Ca^{2+}-ATPase, and myosin ATPase. Here, ATP synthesized by CK is preferentially used to fuel highly energy-demanding processes such as sodium retention, cardiovascular contractility, as well as remodeling of arteries, promoting high blood pressure.[1-6] We showed in a random, multi-ethnic population sample that plasma CK activity after rest, a surrogate measure of tissue CK, is a main predictor of blood pressure, with a crude increase in blood pressure of 14 mm Hg systolic per log CK increase.[2] Although plasma and tissue CK activity were found to be higher in men, persons of African ancestry and obese patients,[2,7] the association was independent of sex, body mass index, or ethnicity, and has been replicated by others.[2,4]

Importantly, in accordance with a causal relationship, evidence indicates that high tissue or resting plasma CK precedes hypertension in animal models and in humans.[6,8,9] In addition, intracellular CK inhibition substantially reduces contractility of human resistance arteries ex vivo.[3] Furthermore, vascular CK gene expression is strongly associated with clinical blood pressure in humans,[5] and high resting plasma CK is found to be a main predictor of failure of antihypertensive therapy in the general population.[10] Thus, CK inhibition might lower blood pressure. Recently, we showed in a randomized control trial that the creatine analogue and competitive CK inhibitor beta-guanidinopropionic acid (GPA), significantly reduced blood pressure in spontaneously hypertensive rats.[11] GPA is synthesized in the kidney in vivo, and elaborate studies in animals of different species and human cell lines indicate the safety profile[12,13] Although the substance is used by sportspersons to increase stamina and lose weight, to our knowledge, there are no human data available for this potential blood pressure lowering agent. Therefore, we assessed the tolerability of a sub-therapeutic dose of GPA in healthy men during one week.

Methods

Trial design

The full study protocol with detailed study procedures has been published previously (please view Supplement 1).[12] In brief, we conducted a randomized, placebo and active controlled, triple blind, parallel group, single center exploratory clinical trial, with three arms: GPA, creatine, and placebo. Trial location was the Academic Medical Center of the University of Amsterdam, the Netherlands. The publication of this trial adheres to the Consolidated Standards of Reporting Trials (CONSORT) 2010 statement.[14]

Participants

We included healthy, non-smoking, non-vegetarian men aged 18 to 50 years, with a normal, non-obese body mass (BMI 18.5 to 29.9 kg/m^2). Inclusion period was from March 2014 to March 2015. Exclusion criteria included high blood pressure defined as systolic ≥140 mmHg or diastolic ≥90 mmHg or the use of antihypertensive drugs, (history of) cardiovascular disease including transient ischemic attack and stroke; the use of plasma CK-increasing drugs including statins; use of acetylsalicylic acid or nonsteroidal anti-inflammatory drugs in the two weeks prior to the first visit; neuromuscular or endocrine disorders; vasculitis; HIV infection; infectious hepatitis; personal or family history of bleeding disorders; sickle cell anemia or other hereditary anemia; current use or use within two months prior to start of the trial of creatine or other guanidino compounds; and abnormalities in glucose, lipid spectrum, thyroid, kidney, or liver biochemistry parameters in the plasma. To stabilize and standardize plasma CK activity during the trial, participants were instructed to refrain from intensive physical exercise three days prior to the baseline visit or during the intervention in the first week.[2,12] All study participants gave written informed consent, and the full study protocol was approved by the hospital's Medical Ethics Review Committee (MERC reference number 38368.018.12).[12] The study complies with the Declaration of Helsinki (64th World Medical Association, General Assembly, Fortaleza, Brazil, October 2013).

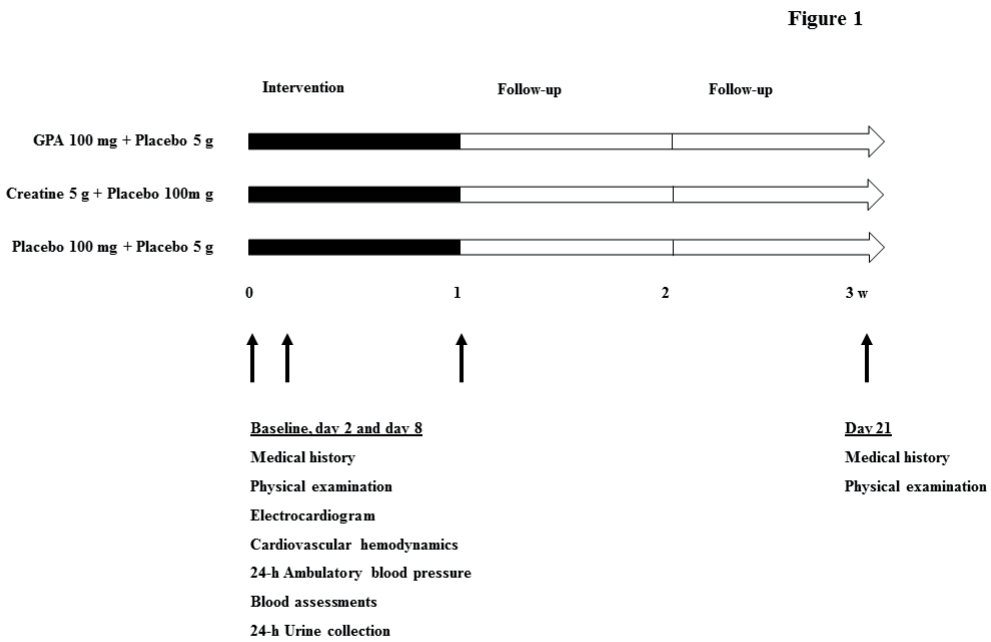
Intervention

Trial procedures are summarized in Figure 1.[12] In brief, participants came to the hospital in the morning after an overnight fast, in 5 visits during 8 days, when medical history was obtained and physical and laboratory examinations were performed. To ensure intervention adherence, trial supplements were ingested by the volunteer in the presence of the trial staff during the hospital visits. In addition, we used pill counts for the supplements ingested at home. At visit 1 (day 0), after baseline measurements (hemodynamic and laboratory assessments) participants were included and randomized. On the next day at Visit 2 (day 1, first intake of trial supplements), participants ingested the randomized, blinded intervention of GPA 100 mg, creatine 5 g, or placebo during the visit. At visit 3 (day 2 of trial supplements), hemodynamic and laboratory tests were performed, trial supplements were ingested, and participants received trial supplements to ingest at home at day 3, 4,5, and 6 after an overnight fast. The participants returned to the hospital for visit 4 at day 7 of the intervention, and ingested the final trial supplements during the visit. They returned for visit 5 on day 8 for hemodynamic measurements and laboratory tests. The 6th and last visit was at day 21, for the final assessment of tolerability.

GPA

GPA or N-(aminoiminomethyl)-beta-alanine; ($Cq_4H_9N_3O_2$), is a structural isomer of creatine.[12,13] GPA is generated in vivo in the kidney via transamidination of β-alanine. The physiological concentration in human plasma is reported to range from trace amounts to 1.40 µmol/L. Clearance is probably renal, akin to creatine, creatinine, and other

Figure 1. Trial overview



Legend. The duration of the intervention was 7 days. The trial supplements started at day 1, after baseline measurements, inclusion, and randomization at day 0 (baseline). After 24 h and after 7 days of trial supplements (day 8), the baseline measurements were repeated. The last visit was at day 21, to assess potential side effects.[12]

guanidino compounds. GPA acts as a competitive inhibitor of cellular creatine uptake, and attenuates the flux through the cytoplasmic creatine kinase reaction. The effect on animal models, using different species, and human cells has recently been summarized.[13] Briefly, GPA decreased intracellular creatine and phosphocreatine in all tissues studied. In skeletal muscle, this effect induced a shift from glycolytic to oxidative metabolism, increased cellular glucose uptake and increased fatigue tolerance. In heart tissue this shift to mitochondrial metabolism was less pronounced. Myocardial contractility was modestly reduced, including a decreased ventricular developed pressure, albeit with unchanged cardiac output. In brain tissue adaptations in energy metabolism resulted in enhanced ATP stability and survival during hypoxia.[13] Despite the lack of human data on efficacy and side effects, GPA is available as a food-supplement, and is used by sportspersons to induce endurance capacity and promote weight loss.[12,13]

Manufacturing and testing

GPA and creatine were considered by the MERC to be food supplements as previously described.[12] GPA was ordered at Sequoia (Sequoia Research Products, Oxford, UK). GPA, creatine, and identical placebo capsules were manufactured by the Pharmacy & Pharmacology Department of the Slotervaart Hospital, Amsterdam, The Netherlands. This department is GMP certificated (ISO 9001:2001). The substance was tested for purity and for cyanide compounds. We established in our certified tests a purity of more than 99% (detection limit) and a cyanide level lower than 1 p.p.m. (detection limit).[12]

Creatine

Creatine, which has an identical molecular formula as GPA, was chosen to assess the effect of the synergist. The average daily rate of creatine synthesis in healthy omnivorous males is estimated to be 1.3 g.[12] We used 5 g as recommended in studies on creatine supplementation. No side effects are apparent at this dose.[12]

Dose calculation

We followed the Food and Drugs Administration (FDA) “Guidance on Estimating the Maximum Safe Starting Dose in Initial Clinical Trials in Adult Healthy Volunteers”, to calculate the maximum recommended starting dose for this first-in-human clinical trial.[15] The purpose of this process is to ensure the safety of the human volunteers. Toxicity should be avoided at the initial clinical dose. However, doses should be chosen that allow reasonably rapid attainment of phase 1 trial objectives. The major elements of this process are as described previously:[12,15]

- Determination of the no observed adverse effect levels (NOAELs) in the tested animal species
- Conversion of NOAELs to human equivalent doses (HED)
- Application of a safety factor

No observed adverse effect level (NOAEL) determination

In animal studies, GPA was administered through the diet in concentrations of 1% or more over 8 weeks without apparent adverse effects. In animals weighing 200 grams and eating 20 grams per day, we calculated a "no observed adverse effect level" of 1,000 mg/kg/day. Furthermore, in a patent application, Meglasson et al. recommended a human dose of 1 to 500 mg/kg/day based on his research in mice and rhesus monkeys. In this paper, rhesus monkeys weighing 9 kg were treated with oral GPA 48 mg/kg/day (432 mg per monkey per day) over 2 weeks without apparent adverse events.[12]

Conversion of the no observed adverse effect level (NOAEL) to human equivalent dose (HED)

We converted the oral NOAELs in rats and monkeys (resp. 1,000 mg/kg/day and 48 mg/kg/day) to oral HEDs based on an algorithm proposed by the FDA based on body surface area. This algorithm proposes a conversion factor from rat to human of 0.16 times the rat dose; and of monkey to men of 0.32 the monkey dose (in mg/kg/day; for a man of 60 kg) resulting in HEDs of resp. 160 mg/kg/day and 15 mg/kg/day for a man of 60 kg.[12]

Application of a safety factor

A safety factor should be applied to the HED to increase assurance that the first dose in humans will not cause adverse effects. The use of the safety factor is based on the possibility that humans may be more sensitive to the toxic effects of a substance than predicted by the animal models, that bioavailability may vary across species, and that the models tested do not evaluate all possible human toxicities, or cannot be expressed by animals or easily measured, such as headache or nausea. We conservatively chose 15 mg/kg/day oral dose for our final calculations of the human dose, because this is the lowest dose, and because of the closer allometric relationship between monkey and man. FDA advises a safety factor of at least 10. Based on an average weight of a male volunteer of 75 kg, we calculated a starting oral dose for this phase 1 study of 75*1.5 mg/day = 112.5 mg/day; we will use 100 mg/day.[12]

Tolerability Health Questionnaire

The participants received a questionnaire to assess tolerability at home during the week of intervention and in the 2 weeks after the intervention. The questionnaire encompassed the perceived side effects of the trial supplements, using check boxes and free text space.[12]

Hemodynamics and Electrocardiography

On day 0 (baseline), day 1 (in the first hour after intake trial supplements), day 2 (after 1 day of trial supplements), and day 8 (after 7 days of trial supplements) of the intervention period, we measured sitting brachial systolic and diastolic blood pressure with an Omron M4 oscillometric device (Omron Healthcare Europe BV, Hoofddorp, the

Netherlands) after 5 minutes of rest with an adjusted cuff size on the left arm, at heart level. In addition, we performed electrocardiography, (MAC 5000 Resting ECG System; GE Healthcare; Boston, MA, USA), and ambulatory 24-hour blood pressure monitoring (Spacelabs 90217 Ambulatory Blood Pressure Monitor, Spacelabs Inc. Redmond, WA, USA). Furthermore, at baseline, day 2, and day 8, we estimated pulse wave velocity and the augmentation index of the aorta in duplicate after 10 min of supine rest (the mean of these two pulse wave velocity measurements was used for the analysis) with the Arteriograph (Tensiomed Kft, Budapest, Hungary). Finally, we monitored haemodynamics including heart rate, cardiac contractility, cardiac output, and total peripheral resistance noninvasively during 5 minutes in the supine position, using a Nexfin BMEYE device (Amsterdam, the Netherlands).

Laboratory studies

At baseline, we assessed resting plasma CK (after 3 days of rest), glucose, lipid profile, creatinine, liver enzymes (ASAT, ALAT), gamma GT, cardiac troponin, TSH (to exclude subclinical hypothyroidism associated with high plasma CK), sodium, potassium, platelet count, coagulation tests (aPTT, PT), and ADP-induced platelet aggregation (area under curve at final concentrations ADP, please see below). Furthermore, we assessed creatinine, sodium, and potassium in 24-h urine. Tests were repeated after 7 days of trial supplements at day 8. Plasma GPA was measured as described previously16 in participants using GPA vs placebo at baseline vs day 8. With the focus on tolerability, further pharmacokinetic studies were considered an unnecessary burden for the participant by the local MERC in this “phase 0” study.[12]

Platelet aggregation test

We assessed ADP-induced platelet aggregation by light transmittance aggregometry (PAP-8E platelet aggregation profiler, Bio/Data Corporation, Horsham, PA, USA) at baseline and after 7 days of intervention. Citrate-anticoagulated blood (0.32%) was centrifuged (Rotina 420R, Hettich Lab Technology, Tuttlingen, Germany) during 15 minutes at 180 g to obtain platelet rich plasma. Platelet poor plasma was prepared by 10 minutes centrifugation at 1550 g. Experiments were performed at 37°C under stirring conditions. Thrombin receptor-activated peptide (TRAP; final concentration 15 µmol/L, Bachem, Bubendorf, Switzerland) was used to induce maximum platelet aggregation (100%). ADP and arachidonic acid were used to initiate the platelet aggregation in the test, final concentrations ADP (0.1, 0.2, 0.5, 1.0, 2.0 µmol/L, Sigma-Aldrich, St. Louis, MO, USA); arachidonic acid (2 mmol/L, Sigma-Aldrich, St. Louis, MO, USA). Aggregations were performed with and without the addition of phosphocreatine (CrP 5 mmol/L final concentration; Sigma-Aldrich, St. Louis, MO, USA).

Adverse and serious adverse events

We did not expect a significant difference in adverse effects between sub-therapeutic GPA and placebo. Although used by sportspersons,[12] there are no FDA or other reports in formal or informal sources on the side effects of ingestion of this dose of GPA in animals

or humans. Adverse events were to be reported by the included subject spontaneously or through the questionnaire, or observed by the trial staff or health care worker, and when occurred, were to be recorded and judged by the study group and the independent physician. The trial staff was to convert the reported symptoms to a standard lexicon, the Common Terminology Criteria for Adverse Events (CTCAE),[17] to facilitate international scientific reporting. Adverse effects were to be classified based on the FDA guideline,[15] as overt toxicity (for example, clinical signs, macro- and microscopic lesions); surrogate markers of toxicity (for example, plasma liver enzyme levels); or other adverse effects; and we planned to use the adverse drug reaction probability scale[18] to assess the causal relationship between trial supplement use and any reported adverse event.

Outcomes

The primary outcome was the tolerability of one week of 100 mg oral GPA daily, as compared to placebo. Secondary outcomes included the comparison of tolerability with creatine, and the effect of one week of oral GPA on hemodynamic parameters, including peripheral and central blood pressure, and cardiac contractility as compared to creatine and placebo. The tertiary outcome was the effect of GPA on biochemical parameters, including ADP-induced platelet aggregation,[19] compared to creatine and placebo.

Sample size

This is a first-in-human study with GPA, with allometric data available from other species.[12] According to the European Medicines Agency guidelines,[20] we included eight subjects in each arm, to assess the tolerability of GPA versus placebo and creatine over one week.

Randomization

Randomization was performed by an independent party, the Clinical Pharmacy Unit of the Academic Hospital of the University of Amsterdam, using a computer-generated, non-adaptive, restricted randomization scheme with a 1:1:1 allocation ratio. The Pharmacy generated the random allocation sequence. All participants who gave consent for participation and who fulfilled the inclusion criteria were randomized to receive either GPA 100 mg with creatine placebo matching active creatine 5 gram; or creatine 5 gram with GPA placebo matching GPA 100 mg; or double dummy placebo over 1 week. The participant received the blinded, randomized trial supplements from the pharmacy. Allocation concealment was ensured, as the pharmacy stored the allocation list and did not release the randomization code until the data bank had been closed and all outcomes were analyzed. Thus, randomization was conducted without any influence of the investigators, outcome assessors, or participant characteristics. After assignment to interventions, trial participants, trial staff, and the outcome assessor remained blinded to whether the participant was given a placebo or a supplement until after all outcome data had been assessed.

Data analysis

The primary outcome was the tolerability of GPA versus placebo as a descriptive measure, in an intent-to-treat analysis. Because of the small sample size, the distribution of the data could not be formally tested. Since parametric analysis may not be accurate with small sample sizes, and nonparametric analysis may lack power to detect a significant difference, we used parametric statistics for our secondary analysis (i.e. arithmetic mean with SE, unpaired t-tests, and one-way ANOVA with the appropriate post-test with Bonferroni correction); and reanalyzed the data in a sensitivity analysis with non-parametric methods (i.e. median with interquartile range, Mann-Whitney test, or Kruskal-Wallis test with a Dunn's post-test). For the secondary outcome, a two-tailed p value <0.05 was considered to statistical significance. We did not adjust the p values for multiple outcomes, but limited formal statistical testing on non-primary outcomes. The nature of missing data was to be analysed and addressed accordingly, using single imputation with unconditional means for data missing completely ad random, as assessed through inspection and with the Little´s test in SPSS. A sensitivity analysis was to be performed for imputed outcomes. All analyses were performed with SPSS statistical software package for Windows, version 22.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± SE, unless indicated otherwise.

Results

The first participant was randomized in March 2014, and the last follow-up visit was performed in March 2015, as the target population of 24 participants was achieved. Trial flow is depicted in Figure 2. Of 29 volunteers assessed for eligibility, 5 did not meet the inclusion criteria, because of vegetarianism (n=1), high blood pressure at baseline screening (n=1), use of prescription drugs (n=2), and tobacco use (n=1). Twenty four randomized participants received the allocated intervention, and 23 completed the study. One participant dropped out on day 4 in the placebo treatment arm because of an external event in his family. This participant experienced no side effects, including not during a re-challenge with the assigned drug. Baseline and day 8 characteristics of all randomized study participants are shown in Table 1. At baseline, there was no significant difference in mean plasma GPA concentration of participants using GPA vs. placebo, respectively 26.88 (SE 0.00) vs 40.63 (0.01) nmol/L, probably reflecting endogenous synthesis. At day 8, mean plasma GPA was significantly higher in the GPA arm compared to placebo as expected, respectively 213.88 (SE 0.07) vs. 32.75 (0.00) nmol/L, a mean difference of 181.13, 95% confidence interval of the difference 26.53 to 335.72 nmol/L p=0.025.

Tolerability

Low dose GPA was well tolerated. Adverse events, reported in all treatment arms, were minor and mild, and mostly present at baseline, except for an unpleasant taste in the mouth without change in the diet reported by one participant in the placebo arm at day 21 (Table 2). There were no unexpected serious adverse reactions or serious

Table 1. Physical Examination and Laboratory Outcomes

	Baseline			Day 8		
Parameter	**GPA**	**Creatine**	**Placebo**	**GPA**	**Creatine**	**Placebo**
BMI, kg/m2	24.5 (0.7)	22.1 (0.5)	24.2 (0.6)	24.4 (0.7)	22.3 (0.5)	23.9 (0.8)
Hemoglobin, mmol/L	8.5 (1.1)	7.6 (4.1)	8.3 (3.0)	9.5 (0.1)	9.6 (0.3)	9.2 (0.4)
Platelets, 109/L	241 (17.9)	261 (10.0)	215 (22.0)	246 (19.5)	263 (7.0)	258 (23.7)
Glucose, mmol/L	5.1 (0.1)	4.9 (0.1)	5.0 (0.1)	4.9 (0.1)	4.5 (0.2)	5.0 (0.1)
Creatinine, micromol/L	74.1 (3.4)	75.5 (3.2)	80.6 (3.0)	71.9 (3.4)	80.6 (3.9)	78.6 (4.1)
Creatine Kinase, U/L	415.6 (198.4)	178.5 (70.2)	341.3 (149.5)	159.9 (38.4)	121.3 (7.8)	152.7 (51.0)
Gamma GT, U/L	35.4 (9.0)	16.4 (1.6)	24.8 (5.6)	40.0 (9.7)	16.1 (1.6)	22.1 (5.3)
ASAT, U/L	34.5 (10.1)	23.8 (1.7)	30.6 (5.5)	27.3 (3.4)	23.0 (2.1)	26.3 (2.3)
ALAT, U/L	32.8 (8.9)	23.5 (3.9)	30.3 (3.9)	35.5 (8.9)	19.1 (1.1)	27.1 (3.9)
Cholesterol, mmol/L	4.6 (0.2)	4.0 (0.2)	4.2 (0.1)	4.7 (0.2)	4.1 (0.2)	4.3 (0.2)
HDL, mmol/L	1.3 (0.1)	1.3 (0.1)	1.4 (0.1)	1.3 (0.1)	1.3 (0.1)	1.3 (0.2)
LDL, mmol/L	2.7 (0.2)	2.3 (0.2)	2.5 (0.2)	2.8 (0.2)	2.4 (0.2)	2.5 (0.2)
Triglycerides, mmol/L	1.1 (0.2)	0.8 (0.2)	0.9 (0.2)	1.4 (0.4)	1.0 (0.2)	1.3 (0.3)
Troponin, µg/L	0.007 (0.0)	0.005 (0.0)	0.009 (0.0)	0.007(0.0)	0.007 (0.0)	0.006 (0.0)
Urine Na, mmol/24h	140.3 (21.8)	127.5 (18.9)	182.4 (12.6)	171.4 (27.2)	109.5 (19.7)	162.0 (20.2)
Urine K, mmol/24h	67.8 (10.3)	75.0 (11.1)	82.0 (12.6)	67.4 (9.6)	58.4 (26.1)	77.6 (14.3)
Urine Creatinine, µmol/kg/24h	118.0 (35.1)	187.2 (44.6)	134.1 (34.8)	144.3 (32.7)	135.7 (32.3)	113.6 (37.6)

Legend. Data are mean (SE) for baseline and after 7 days of treatment. Biochemistry data are plasma unless stated otherwise. Data concern n=8 men in each treatment arm, except for placebo, which was n=7 at day 8. Mean age in the 3 treatment arms was respectively 27.4; 22.8; and 25,8 y. Participants had significant lower BMI and cholesterol in the creatine treatment arm from baseline compared to the GPA arm. There were no significant differences at day 8 between treatment arms. BMI, Body mass index.

adverse events. No significant changes were found compared to placebo in clinical safety parameters, physical examination including blood pressure, or laboratory measurements. In addition, there were no significant differences in 12-lead ECG parameters after treatment including an unchanged QT-interval.

Hemodynamics

The hemodynamic parameters are presented in Table 3. At baseline participants had significant higher aorta augmentation index in the GPA treatment arm compared to the creatine and placebo arm (p=0.015). There were no significant differences between treatment arms after 7 days of active treatment.

Platelet aggregation test

There was no significant difference between GPA, creatine and placebo in platelet aggregation parameters at baseline or at day 8.

Table 2. Reported adverse events at baseline and during follow up

Day	GPA (N=8)	Creatine (N=8)	Placebo (N=8)
0	**n=4 participants** Cough (3) Fatigue (2) Insomnia (1) Headache (1) Hyperhidrosis (1)	**n=6 participants** Cough (2) Fatigue (5) Dizziness (1) Headache (2) Hyperhidrosis (2) Myalgia (1)	**n=5 participants** Cough (3) Fatigue (3) Insomnia (1)
1	**n=3 participants** Cough (2) Restlessness (1)	**n=4 participants** Cough (2) Fatigue (1) Dizziness (1) Headache (2) Hyperhidrosis (2)	**n=3 participants** Cough (2) Restlessness (1)
3	**n=1 participant** Cough (1)	**n=1 participant** Fatigue Restlessness (1)	**n=3 participants** Cough (3) Fatigue (1)
7	**n=1 participant** Cough (1)	**n=1 participant** Fatigue Restlessness Cough Hyperhidrosis Headache Myalgia (1)	**n=2 participants** Cough (2)
21	**n=1 participant** Cough (1)	**n=1 participant** Fatigue Restlessness Headache (1)	**n=2 participants** Cough (1) Fatigue (2) Insomnia* (1) Dysgeusia (1)

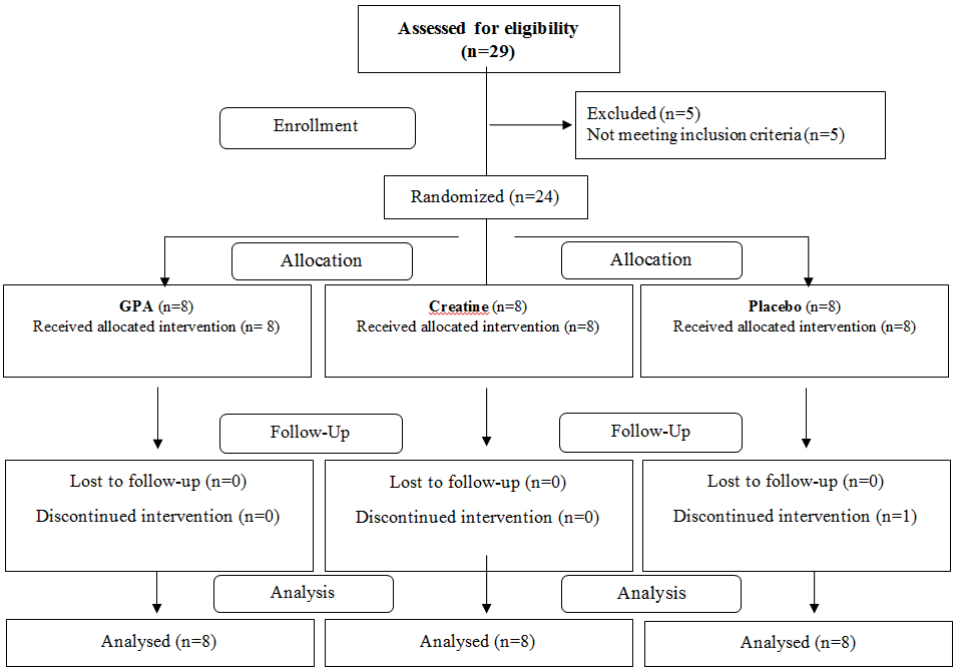
Legend. Data Treatment allocation, data collection and data analysis were blinded. Day 0, baseline; 1, 3, 7, day of active treatment; 21, 2 weeks after active treatment. Data between brackets are number of participants with adverse effects. All reported adverse effects were CTCAE[14] classification (mild), except insomnia* in the placebo treatment arm, which was moderate.

Discussion

The main finding of this first-in-human study is that GPA, given during 1 week in a sub-therapeutic dose as recommended by the FDA,[15] is safe and well tolerated in healthy men. There were no serious or severe adverse events reported with the use of GPA, and no significant differences with placebo in safety measures including self-reported data obtained with unstructured and structured questionnaires, physical examination, laboratory tests including kidney and liver parameters, or cardiovascular safety including the QT interval.

This study was conducted because incremental data indicate that the ATP regenerating enzyme CK enhances the energy demanding processes involved in hypertension.[1-6] The CK enzyme system is thought to promote hypertension through rapid regeneration of

Figure 2. CONSORT Flow Diagram



Legend. Participant's flow in the study.
All eight participants in the placebo arm, including one drop out, were analysed for the primary outcome of tolerability of GPA vs placebo.

ATP from phosphocreatine near ATPases involved in resistance artery contractility and salt retention.[1-6,10] Animal and human studies found that plasma and tissue CK activity was a main determinant of blood pressure and of resistance artery contractility, while hypertension was found to be more severe in individuals with high plasma CK activity.[1-6,10] In addition, the creatine analogue and competitive CK inhibitor GPA safely and reversibly reduced blood pressure in spontaneously hypertensive rats.[11] Therefore, the CK enzyme system is a potential target for lowering blood pressure in humans with high plasma CK activity.

Currently, there is a need for new conservative options to treat resistant hypertension.[10,21-24] A substantial proportion of treated hypertensive patients do not achieve blood pressure control, even with multiple drugs. Risk factors for poor control include obesity, age, African ancestry, the presence of diabetes or end organ damage; but non-adherence of the patient, the white-coat effect, therapeutic inertia of the physician, dietary factors, or the concomitant use of blood pressure increasing drugs may also contribute.[10,21-24] Importantly, resting plasma CK was the main predictor of failure of hypertension treatment in the general population.[10] Therefore, antihypertensive agents acting through CK inhibition might aid in achieving better control in patients with difficult-to-treated hypertension and high plasma CK activity.[10]

Table 3. Cardiovascular Assessments

	Baseline			Day 8		
Parameter	**GPA**	**Creatine**	**Placebo**	**GPA**	**Creatine**	**Placebo**
24-hours SBP, mmHg	124 (2.2)	122 (2.9)	123 (3.0)	127 (2.9)	126 (2.5)	120 (2.3)
24-hours DBP, mmHg	72 (1.3)	69 (2.1)	71 (2.4)	73 (1.7)	72 (3.0)	71 (1.9)
24-hours HR, bpm	74 (3.8)	72 (4.4)	68 (2.7)	75 (3.7)	75 (4.7)	66 (3.3)
Sitting SBP, mmHg	127 (1.8)	125 (2.0)	122 (3.9)	128 (2.8)	120 (4.5)	124 (3.7)
Sitting DBP, mmHg	72 (6.4)	72 (2.3)	73 (3.5)	72 (2.5)	71 (3.5)	72 (3.3)
Sitting HR, bpm	72 (4.1)	65 (13.2)	61 (4.4)	77 (5.2)	73 (5.8)	62 (4.2)
Supine SBP, mmHg	116 (8.3)	117 (3.9)	113 (3.4)	106 (3.6)	104 (7.8)	109 (5.4)
Supine DBP, mmHg	66 (5.2)	64 (1.7)	61 (3.4)	62 (2.6)	59 (3.6)	62 (2.6)
Supine HR, bpm	68 (4.3)	61 (4.6)	61 (3.3)	70.5 (4.8)	68 (4.3)	61 (3.2)
Stroke volume, mL	111.1 (5.8)	106 (12.7)	115.8 (5.6)	108 (3.8)	108.4 (6.8)	118 (4.1)
Cardiac output, L/min	7.4 (0.3)	15.1 (9.0)	7.1 (0.5)	7.5 (0.4)	7.2 (0.4)	7.2 (0.4)
LVC (dP/dt), mmHg/s	819.6 (117.5)	1068.4 (147.9)	757.4 (89.3)	697.3 (35.4)	761 (88.0)	820.4 (71.4)
SVR, dyn·s/cm5	956.6 (84.3)	1407.9 (463.1)	921.3 (60.2)	831.1 (68.8)	869.3 (57)	911.6 (72)
PWV, m/s	5.9 (0.5)	6.0 (0.4)	6.3 (1.0)	5.9 (0.4)	6.4 (0.6)	5.3 (0.3)
Aix aorta, %	14.5 (4.9)*	1.2 (1.5)*	4.3 (2.3)*	4.1 (2.1)	5.1 (5.5)	6.9 (2.4)
QT-interval, ms	382 (8.5)	398 (15.2)	403 (6.9)	380 (10.5)	398 (11.5)	405 (9.4)

Legend. Data are mean (SE) for baseline and after 7 days of treatment. N=8 in each treatment arm, except for placebo N=7 at day 8. SBP, Systolic blood pressure; DBP, Diastolic blood pressure; HR, heart rate; bpm, beats per minute; LVC, left ventricular contractility; SVR, Systemic vascular resistance; PWV. Pulse wave velocity; Aix, Augmentation index of aorta. PWV N=6-8; AIX N=5-8. Participants assigned to GPA had significant higher Aix from baseline compared to creatine and placebo treatment arm (* p=0.015). There were no significant differences at day 8 between treatment arms.

Data of more than 120 animal studies in different species indicate that creatine is not indispensable, as recently summarized.[13,25] Taking the wide variation in CK activity found in humans into account,[1,2,6,26-28] we suggest that moderate CK inhibition should be feasible in humans with high CK to reduce blood pressure without major side effects.[12]

The main strength of this study is that we provide first-in-human data on the safety and tolerability of the specific small molecule CK inhibitor GPA, in a sub-therapeutic dose given during one week, in a triple blinded randomized, placebo and active controlled trial. We collected these data in close adherence to the US FDA and European guidelines.[15,20] Limitations are the obligatory sub-therapeutic dosing and the use during one week, aimed at preventing toxicity, which limited efficacy assessments. Another limitation is that we did not assess pharmacokinetics of GPA, following the imperative advice of our local medical ethical committee to focus on safety and tolerability in this first-in-human data collection. Finally, although tolerability studies are part of the formal assessment of new drugs, the relevance of such studies for clinical safety is limited, mainly because of the small samples sizes. Thus, the value of tolerability studies for drug development has been questioned.[29] However, even with larger studies, no drug can be universally acclaimed to be well tolerated and safe.[29] We perceive that the presented data on GPA

should be looked upon within the context of the existing evidence, the molecule is not novel but kidney-synthesized, has a large body of data collected in different animal species and human cells, is on the market as a food supplement,[12] and was tested in this study only during one week in a sub-therapeutic dose in healthy men. We observed an increase in plasma GPA concentration, without evidence of any desirable or undesirable effect. Further clinical studies are needed to establish early in the process of further drug development the safety, tolerability, and pharmacokinetics of GPA doses or plasma concentrations likely to produce a pharmacological effect.

Conflict of interest

L.B. is an inventor on NL patent WO/2012/138226 (filed).

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Contributors

FK co-designed and conducted the clinical trial, conducted the primary statistical analysis, and drafted the manuscript. DH co-designed the platelet tests and participated in conducting the clinical studies. YH co-designed and conducted the clinical trial. LW co-designed and conducted the clinical trial. MS conducted the platelet tests. IO co-designed and supervised the clinical trial. GM co-designed and supervised the clinical trial. RN co-designed and supervised the platelet tests. GS co-designed the clinical trial and assessed GPA. JC co-designed the clinical trial. LB, the grant holder, designed the clinical trial and platelet studies, provided statistical expertise, supervised the clinical and laboratory studies, conducted and supervised the statistical analyses, and drafted the manuscript. All authors contributed to the writing of the manuscript for important intellectual content, and read and approved the final manuscript.

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Chapter 8

Summary and General Discussion

Summary and General Discussion

An introduction for this thesis is provided in **Chapter 1**. There is increasing evidence that plasma creatine kinase (CK) levels are associated with hypertension[1-17] and with resistance to treatment.[6-8] However, the underlying mechanisms of such associations are unknown, and it remains to be established whether creatine kinase is causally involved in hypertension. It was proposed that enhanced resistance artery contractility due to high vascular smooth muscle creatine kinase-BB activity plays a causal role, by increasing total peripheral resistance.[2,10] Accordingly, the first part of this thesis addresses CK expression and the effect of inhibition of CK on contractility in human resistance arteries. The second part investigates the possibility of interfering with CK activity to treat hypertension. In this chapter we summarize the main findings, discuss the involved mechanisms and evaluate the clinical perspectives.

Summary

Part I. Creatine kinase, resistance artery contractility and blood pressure

The studies in this part focus on the association between high tissue and plasma CK activity in resistance artery contractility and blood pressure. First, we systematically review the evidence in **Chapter 2** on the association between this new risk factor CK and blood pressure outcomes. We used a narrative synthesis approach and conducted a systematic search to include studies on non-pregnant adult humans that address the association between plasma CK and blood pressure outcomes. We searched electronic databases and performed hand search without language restriction. We investigated the association between CK and blood pressure outcomes as continuous measures. Other outcomes included the association between CK and blood pressure categories (normotension and hypertension, subdivided in treated controlled, treated uncontrolled and untreated hypertension). We retrieved 139 reports and included 11 papers from 10 studies assessing plasma CK activity in 34,578 participants, men and women, of African, Asian, and European ancestry, aged 18 to 87 y. In 9 reports, CK was associated with blood pressure levels, hypertension (vs normotension), and/or treatment failure. The adjusted increase in systolic blood pressure (mmHg/log CK increase) was reported between 3.3 [1.4 to 5.2] and 8.0 [3.3 to 12.7]; the odds ratio of hypertension with high vs low CK ranged between 1.2 and 3.9; and CK was a predictor of treatment failure in the general population, with an adjusted odds ratio of 3.7 [1.2 to 10.9]. **Chapter 3** reports the association between resistance artery CK gene expression and blood pressure. We isolated resistance-sized arteries from omental fat donated by women who consecutively underwent uterine fibroid surgery. 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 isoenzyme 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 (DBP) (correlation coefficient,

0.9; 95% confidence interval, 0.6-1.0) and were well correlated with systolic blood pressure (SBP), 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. This is the first direct evidence suggesting that resistance artery creatine kinase mRNA expression levels concur with blood pressure levels, almost doubling with hypertension. This adds to the existing evidence on the potential role of CK in the enhancement of vascular contractility and pressor responses. In **Chapter 4**, we assess the effect of creatine kinase inhibition on contractile properties of human resistance arteries. Nineteen consecutive women, mean age 42 years (SE 1.3), mean systolic/diastolic blood pressure respectively 142.6 (SE 5.9)/85.6 (3.4) mm Hg (9 hypertensive), donated an omental fat sample during abdominal surgery. We compared vasodilation after the specific CK inhibitor 2,4-dinitro-1-fluorobenzene (DNFB; $10(^{-6})$ mol/l) to sodium nitroprusside ($10(^{-6})$ mol/l) in isolated resistance arteries using a wire myograph. Additionally, we assessed predictors of vasoconstrictive force. DNFB reduced vascular contractility to 24.3% (SE 4.4), $P < 0.001$, compared to baseline. Sodium nitroprusside reduced contractility to 89.8% (SE 2.3). Maximum contractile force correlated with DNFB effect as a measure of CK ($r = 0.8$), and with vessel diameter ($r = 0.7$). The increase in contractile force was 16.5 mN [9.1-23.9] per unit DNFB effect in univariable and 10.35 mN [2.10-18.60] in multivariable regression analysis. This study extends on our previous findings in pregnant normotensive women of CK-dependent microvascular contractility, indicating that CK contributes significantly to resistance artery contractility across human normotension and primary hypertension also outside the context of pregnancy.

Part II. Creatine kinase and therapeutic implications

In **Part II** we investigate whether modulation of the CK system might become a target for blood pressure lowering in humans with high plasma CK activity and difficult-to-treat hypertension. In **Chapter 5** we first assess whether inhibiting the creatine kinase system with a specific blocker, safely reduces blood pressure (BP) in the spontaneously hypertensive rat. In a 4-week randomized controlled trial, male 16-week-old spontaneously hypertensive rats (N=16) were randomly assigned to the specific competitive creatine kinase inhibitor beta-guanidinopropionic acid (GPA), 3% supplemented chow vs. standard chow. Creatine kinase inhibition reduced BP safely and reversibly. Mean baseline BP of, respectively, 191.5 (standard error 4.3) mmHg SBP and 143.1 (4.1) mmHg DBP was reduced by, respectively, 42.7 (5.5) mmHg SBP and 35.6 (5.0) mmHg DBP ($P<0.001$) compared with controls, with evidence of enhanced vasodilation and a diuretic effect. This is the first report on the BP-lowering effect of creatine kinase inhibition. Our data indicate that modulation of the creatine kinase system might become a novel treatment for hypertension. Therefore in **Chapter 6** we developed a protocol for a first-in-man trial with the specific CK inhibitor beta-guanidinopropionic acid (GPA) in healthy man. In **Chapter 7** we report that beta-guanidinopropionic acid was well tolerated in a randomized placebo controlled trial. The interventions consisted of one week daily oral administration of GPA in a subtherapeutic dose of 100mg, creatine 5 gram, or placebo. Twenty four randomized participants received the allocated intervention. There were no serious or severe adverse events with GPA, creatine and

placebo after 1 week of active treatment. No abnormalities were reported from physical examination, laboratory determined toxicity including kidney and liver parameters, or cardiovascular safety including QT interval between treatment arms. These are the first human data of the specific CK inhibitor and potential new blood pressure lowering agent GPA. We found no evidence of toxicity with subtherapeutic doses. The next step would be a dose-escalation trial to assess safety and tolerance in higher doses in healthy volunteers.

General Discussion

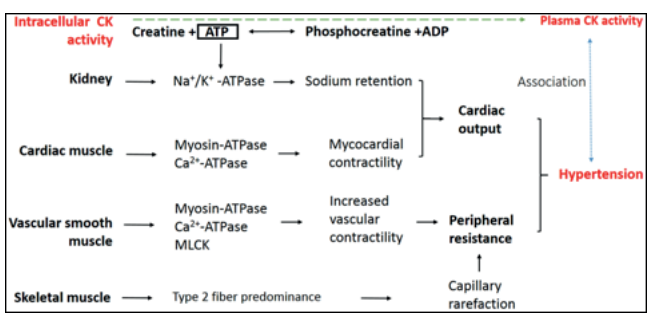
This thesis is based on the central hypothesis that higher intracellular CK activity in a range of cells leads to an increase of blood pressure. In this discussion, we address the rationale of this hypothesis, the evidence from this thesis and earlier work in favor of or against this hypothesis, the steps required to further substantiate this, the relevance of circulating plasma CK levels as a valid proxy for intracellular CK activity, and the possibilities for antihypertensive treatment based on interfering with intracellular CK activity.

Mechanisms linking intracellular creatine kinase activity to blood pressure

Figure 1 summarizes possible mechanisms that link increased CK activity in vascular smooth muscle cells, cardiomyocytes and renal tubular epithelial cells to higher systemic arterial blood pressure levels. Systemic blood pressure equals the product of cardiac output and total peripheral resistance. Accordingly, a higher peripheral resistance, cardiac contractility and excessive sodium retention increase blood pressure. Here we briefly discuss the involvement of CK in cardiac contractility and renal sodium retention, and elaborate on peripheral resistance and small artery contractility, which formed the major subject of this thesis.

Cardiac contractility

Figure 1. Proposed mechanisms through which creatine kinase may lead to hypertension



Legend: Proposed mechanisms through which the high creatine kinase (CK) phenotype with high activities in skeletal muscle, heart, kidney, and smooth muscle, may lead to hypertension. In the kidney, high CK activity may lead to increased sodium retention through increased ATP availability for Na^+/K^+-ATPase, leading to a higher cardiac output. In the cardiovascular system, high CK activity is thought to provide ATP to enzymes involved in contractile responses, including myosin ATPase, Ca^{2+}- ATPase, and myosin light chain kinase (MLCK), leading to increased peripheral resistance of blood vessels. In addition, high CK activity in type 2 fibers is associated with capillary rarefaction and increased peripheral resistance.

In the heart, the CK-system is of particular importance for the maintenance of local ATP levels and contribution to myocardial contractile capacity.[1,11,17,21] Myofibrillar CK, functionally coupled to myosin ATPase, maintains high ATP/ADP ratios, which prevents a decline in maximum shortening velocity of the myofibrils.[21] There is compelling evidence from mostly animal studies that differences in CK activity affect cardiac performance in a range of physiological and pathological conditions. An inhibition or decrease of myocardial creatine kinase activity induces a major decline in contractile reserve and a greatly decreased contractile ability, in particular during increased demands. This indicates that the capacity to resynthesize ATP through the creatine kinase system is essential for the utilization of the full dynamic range of myocardial performance.[1,21] Thus, creatine kinase activity in myofibrils is needed to sustain normal tension and relaxation.[1,21] Currently we are studying the relation between left ventricular contractility and plasma CK activity in a large cross-sectional population study.

Renal function

High plasma CK levels are associated with increased sodium reabsorption in the renal tubules.[1,16,17,22,23] Recently, we showed that subjects with high plasma CK activity excrete significantly less sodium after a high salt diet.[16] In the kidney, CK is functionally coupled to renal Na^+/K^+-ATPase and the ATP produced by colocalized CK is preferentially used for the high and fluctuating ATP demand of sodium transport across the tubular epithelial cells.[1,16,17,22,23] CK has been found to be particularly active in the thick ascending limb of Henle's loop and the collecting tubules. [1,16,17,22,23] Thus, high CK activity in the kidney tubule cells may lead to increased availability of ATP for the active process of sodium reabsorption. Therefore, greater creatine kinase activity in renal tubule epithelial cells might enable greater salt retention, possibly leading to higher blood pressures. Here, an underlying assumption is that plasma CK reflects activity in these cells, as will be discussed below.

Peripheral resistance and small artery function and structure

Total peripheral resistance is increased in a range of hypertensive disorders, including primary hypertension.[18] Total peripheral resistance is dictated by the organization of the microvascular beds and the diameters of the individual vessels in the microcirculation.[1,2,17] It should be noted that small changes herein have major effects on resistance, due to the fourth power relation of vascular resistance on diameter, as formulated by the Poiseuille law. [1,2,17] Thus, a small increase in microvascular CK activity and contractility might markedly increase peripheral resistance to blood flow, with a potentially large impact on blood pressure.[1,2,17] Therefore, these mechanisms may contribute to greater hypertension risk in those with high CK.

In **Chapter 3** we showed that CK mRNA in isolated human resistance arties strongly correlates with clinical blood pressure, while **Chapter 4** demonstrated that vascular contractility is highly CK-dependent. The fact that CK is involved in human small artery contractility and that vascular CK mRNA is raised in hypertension argue in favor of vascular CK-dependent blood pressure. However, it needs to be determined whether upregulation of vascular CK at the transcriptional level is associated with increased

protein expression and activity. Moreover, the causality of the relation between cellular CK and BP needs to be discussed, as follows hereunder.

It is well established that endothelial function is crucial for regulation of vasomotor tone in the resistance vessels.[24] Endothelial function was not the subject of the current thesis. Decking et al. observed expression of mitochondrial Ck and BB-CK in aortic and microvascular endothelial cells and provided evidence that CK contributes to buffering and possible shuttling of energy in these cells.[25] To the best of our knowledge the consequences of altered endothelial CK activity for vascular function have not been studied. In **Chapter 4** we aimed to test the effect of DNFB, a blocker of CK, on endothelial function in human resistance arteries. However, this compound fully blocked the preconstriction, making it impossible to test endothelial-dependent dilation to e.g. acetylcholine. The recently identified intimate relation between endothelial energy metabolism and function shows that such studies are needed.[26]

In hypertension, arteriolar and capillary rarefaction, and remodelling of the resistance vasculature (small arteries) are commonly observed changes that affect the structure of the microvascular network.[17,27,28] However there is no direct evidence that CK is involved in small artery remodelling.[19] High CK activity in skeletal muscle with a predominance of type II fibers is associated with lower capillary density [15,29] and may increase peripheral resistance.

Plasma CK activity as a proxy for intracellular activity

The association between blood pressure and plasma CK was reported in a range of studies, including European, Indonesian, Taiwanese, Indian, and West-African populations across the world, as summarized in **Chapter 2**. In a prospective study, persistent high plasma CK activity at baseline was correlated with SBP and DBP.[30]

The plasma CK concentration results from a balance of release from a range of cells and clearing from the circulation. There is little reason to believe that the plasma concentration or activity is causally involved in BP control. Rather, plasma CK may be a valid proxy for cellular activity in a range of relevant cells. Alternatively, one could argue that increased plasma CK activity results from hypertension, e.g. due to tissue damage. There are arguments for both views. Normal tissue releases CK proportional to the intracellular CK concentration, a physiological process that occurs without tissue damage, as summarized by Brewster et al.[2] Normal tissue loses a small fraction of cytosolic CK into the interstitial space, as was shown in 31P nuclear magnetic resonance spectroscopy studies.[2,8,31] Interstitial CK is subsequently transported through lymphatic vessels into the bloodstream. [2,8,31] Therefore, plasma CK in healthy persons at rest is likely to reflect tissue CK.[2,8,31] However, with exercise, lymphatic flow increases and CK from the interstitial space may enter the circulation rather abruptly, where it is cleared by the liver in around 3 days.[2,8,31] With frank tissue damage, such as after eccentric exercise, where the muscle contracts and stretches at the same time, or with myocardial infarction or brain trauma, large quantities of CK enter the circulation, proportional to intracellular CK and the damaged area.[2,17,32] Thus, the use of plasma CK as a proxy for

cellular activity requires careful conditions and patient selection. Exclusion of a possible contribution of cell damage could be based on the determination of other enzymes in the plasma, such as lactate dehydrogenase, which are not expected to be correlated with cellular CK activity.

Does increased cellular CK activity raise blood pressure?

While this thesis and the above studies provide a case for the association between blood pressure and cellular CK activity in the vascular smooth muscle cells and the myocardium, the order of causality remains an open question. An increase in cellular creatine kinase activity could promote the development of hypertension by increased contractility, thereby maintaining blood pressure at a high level.[1,33] Alternatively, increased cellular creatine kinase activity might be required to meet the higher energy demand due to the augmented cardiac and vascular workload.[5]
We partially addressed this question in **Chapter 5**. Here, we showed in an animal study that hypertension can be treated by inhibition of the CK-system. This strongly contributes to the evidence for a causal role of CK in the development of hypertension. Furthermore, in the SHR, evidence was found that high cardiac tissue creatine kinase activity precedes hypertension in SHR.[5] Thus, although proving causality is difficult, the findings in this thesis call for further exploration of the pathways via which CK affects blood pressure. In animals, further interventional studies are needed. This includes genetic models such as SMC-specific CKB knockouts, which would allow testing the causal role of cellular CK activity in vascular contractility, peripheral resistance and blood pressure development. In humans, a longitudinal study is needed to investigate the relation between CK and BP over time.

Perspectives and future research

Patients with high plasma CK activity have an increased risk to have difficult-to-treat hypertension.[7-9] We reported that serum CK was a main and independent predictor of antihypertensive treatment failure in a cross-sectional population study.[8] Plasma CK activity was significantly higher in treated uncontrolled hypertension than in normotension and treated controlled hypertension.[7-9] In these patients treatment failed in 72.9% of participants within the highest creatine kinase tertile vs. 46.7% within the lowest tertile.[4] Treatment fails in nearly half of hypertensive patients, including many patients with uncomplicated hypertension.[7-9] Therefore, antihypertensives acting via new mechanisms such as GPA might aid in achieving better control in these patients. We have described the promising results of GPA in hypertensive animals and our first-in-man study in **Chapter 5** and **7**. These data suggest that inhibition of the CK-system might become an interesting target for lowering blood pressure. However, several issues need to be addressed before the use of GPA is ready for clinical practise. First, further studies should investigate the efficacy of higher dosages and longer duration of GPA in hypertensive animals. Secondly, research should be extrapolated to humans. Our first-in-man study with GPA showed that GPA was well tolerated in healthy man and was uneventful. The next step in this development process is to conduct a phase I/II dose escalation trial and investigate safety, pharmacokinetics and pharmacodynamics in

healthy volunteers.

In conclusion we studied the role of CK in the development of hypertension, possibly based on the energy needed to create high blood pressure. We conducted laboratory, clinical, and population studies on CK and hypertension in humans, and developed a novel modus of blood pressure lowering in CK inhibition. We look forward to more studies offering a better understanding on this new and exciting area of clinical hypertension.

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Appendices

Nederlandse Samenvatting

Hoofdstuk 1 geeft een inleiding van dit proefschrift. Er is toenemend bewijs dat plasma creatinekinase (CK) activiteit samenhangt met hoge bloeddruk [1-17] en met resistentie tegen behandeling [6-8]. De onderliggende mechanismen van deze samenhang zijn echter onbekend, en het moet nog worden vastgesteld of er een oorzakelijk verband bestaat tussen cellulaire CK activiteit en hypertensie. Verhoogde contractiliteit van weerstandsvaten als gevolg van creatinekinase-BB-activiteit in de gladde spiercellen zou een oorzakelijke rol spelen, via toename van de totale perifere weerstand [2,10]. Het eerste deel van dit proefschrift onderzoekt de expressie van CK en het effect van remming van CK op de contractiliteit in geïsoleerde humane weerstandsvaten. Het tweede deel onderzoekt de mogelijkheid om te interfereren met CK-activiteit om hypertensie te behandelen. Hier vatten we de belangrijkste bevindingen samen.

Deel I. Creatinekinase, weerstandsvaten contractiliteit en bloeddruk

De studies in dit deel richten zich op het verband tussen hoge weefsel- en plasma-CK-activiteit in geïsoleerde humane weerstandsvaten en bloeddruk. Ten eerste bekeken we in **Hoofdstuk 2** systematisch de aanwijzingen over de associatie tussen deze nieuwe risicofactor CK en bloeddruk. We gebruikten een narratieve synthese en voerden een systematische zoekactie uit naar studies naar de associatie tussen plasma-CK en bloeddruk in niet-zwangere volwassen mensen. We hebben elektronische databases doorzocht en handmatig gezocht naar relevante artikelen. We onderzochten in de eerste plaats de associatie tussen CK en bloeddruk als continue maat. Een andere uitkomst was de associatie tussen CK en bloeddrukcategorieën (normotensie en hypertensie, onderverdeeld in behandelde gecontroleerde, behandelde ongecontroleerde en onbehandelde hypertensie). Dit leverde 139 artikelen op en hiervan hebben wij 11 artikelen geïncludeerd met 10 studies naar plasma-CK-activiteit bij 34.578 deelnemers, mannen en vrouwen, van Afrikaanse, Aziatische en Europese afkomst, in de leeftijd van 18 tot 87 jaar. In 9 artikelen was er een verband tussen CK en bloeddrukniveaus, hypertensie (vs normotensie) en / of therapiefalen. De gecorrigeerde stijging van de systolische bloeddruk (toename in mmHg / log CK) was tussen 3,3 [1,4 tot 5,2] en 8,0 [3,3 tot 12,7]; de odds ratio van hypertensie met hoge versus lage CK varieerde tussen 1,2 en 3,9 en CK was een voorspeller van therapiefalen in de algemene populatie, met een gecorrigeerde odds ratio van 3,7 [1,2 tot 10,9]. **Hoofdstuk 3** rapporteert over de associatie tussen CK-genexpressie en de bloeddruk in weerstandsvaten. We isoleerden weerstandsvaten uit buik-vet (omentum) van vrouwen die een operatie ondergingen voor verwijdering van vleesbomen. Weerstandvaten van 13 vrouwen werden geïncludeerd, 6 normotensieve en 7 hypertensieve, gemiddelde leeftijd 42,9 jaar (standaard fout (SE), 1,6) en gemiddelde systolische / diastolische bloeddruk, 144,8 (SE 8,0) / 86,5 (SE 4,3) mm Hg. Arteriolaire creatine kinase iso-enzym mRNA werd vastgesteld met behulp van kwantitatieve real-time PCR. Genormaliseerde creatine kinase B mRNA-kopie aantallen, variërend van 5,2 tot 24,4 (gemiddelde, 15,0; SE, 1,9), vertoonden een bijna perfecte correlatie met diastolische bloeddruk (DBP) (correlatiecoëfficiënt, 0,9, 95% betrouwbaarheidsinterval, 0,6-1,0)) en waren goed gecorreleerd met systolische bloeddruk (SBP), met een 90% relatieve toename van creatine kinase B mRNA van de

weerstandsvaten bij hypertensie vergeleken met normotensie. Genormaliseerde kopie aantallen waren respectievelijk 19.3 (SE, 2.0) versus 10.1 (SE, 2.1), P = 0.0045. Dit is het eerste directe bewijs dat suggereert dat expressieniveaus van CK in weerstandvaten verband houden met bloeddrukniveaus, met een verdubbeling in hypertensie ten opzichte van normotensie. Dit draagt bij aan het bestaande bewijsmateriaal over de potentiële rol van CK en vasculaire contractiliteit. In **Hoofdstuk 4** onderzochten we het effect van creatinekinase-inhibitie op contractiele eigenschappen van humane weerstandsvaten. We includeerden 19 vrouwen, gemiddelde leeftijd 42 jaar (SE 1,3), gemiddelde systolische / diastolische bloeddruk respectievelijk 142,6 (SE 5,9) / 85,6 (SE 3,4) mm Hg (9 hypertensie), die buik-vet van het omentum beschikbaar stelden tijdens abdominale chirurgie. We vergeleken vasodilatatie na de specifieke CK-remmer 2,4-dinitro-1-fluorbenzeen (DNFB; 10^{-6} mol / l) met natrium nitroprusside (10^{-6} mol / l) in geïsoleerde weerstandsvaten met behulp van een zogenaamde draadjes-myograaf. Daarnaast hebben we voorspellers van contractiekracht van de vasculaire gladde spiercellen onderzocht. DNFB verminderde vasculaire contractiliteit tot 24,3% (SE 4,4), P <0,001, vergeleken met de uitgangswaarde. Natrium nitroprusside verminderde de contractiliteit tot 89,8% (SE 2.3). Maximale contractiekracht was gecorreleerd met het DNFB effect als een maat voor CK (r = 0,8) en met vaatdiameter (r = 0,7). De toename van de contractiekracht was 16,5 mN [9,1-23,9] per eenheid DNFB-effect in univariabele en 10,35 mN [2,10-18,60] in multivariabele regressieanalyse. Deze studie gaat verder in op onze eerdere bevindingen bij zwangere vrouwen met normale bloeddruk met CK-afhankelijke microvasculaire contractiliteit, wat aangeeft dat CK significant bijdraagt aan de contractiliteit van humane weerstandsvaten bij normotensie en primaire hypertensie, ook buiten de context van zwangerschap.

Deel II. Creatinekinase en therapeutische implicaties

In Deel II onderzochten wij of modulatie van het CK-systeem een target zou kunnen worden voor bloeddrukverlaging bij patiënten met hoge plasma-CK-activiteit en moeilijk te behandelen hypertensie. In **Hoofdstuk 5** onderzochten we eerst of het remmen van het creatinekinase-systeem met een specifieke antagonist de bloeddruk veilig verlaagde in spontaan hypertensieve ratten. In een gerandomiseerde studie over 4 weken werden 16 weken oude spontaan hypertensieve ratten (N = 16) willekeurig toegewezen aan de specifieke competitieve CK-remmer bèta-guanidinopropionzuur (GPA), 3% gesuppleerde voeding versus standaardvoer. Creatinekinase inhibitie verminderde de bloeddruk veilig en reversibel. De bloeddruk vóór interventie was respectievelijk 191,5 (SE 4,3) mmHg SBP en 143,1 (SE 4,1) mmHg DBP, en nam af met respectievelijk 42,7 (SE 5,5) mmHg SBP en 35,6 (SE 5,0) mmHg DBP (P <0,001) vergeleken met controles, met bewijs van verhoogde vasodilatatie en een diuretisch effect. Dit is het eerste rapport over het bloeddrukverlagend effect van remming van creatinekinase. Onze gegevens wijzen erop dat modulatie van het CK-systeem een nieuwe behandeling voor hypertensie kan worden. Daarom hebben we in **Hoofdstuk 6** een protocol ontwikkeld voor een first-in-man studie met de specifieke CK-remmer bèta-guanidinopropionzuur (GPA) bij gezonde mensen. In **Hoofdstuk 7** rapporteren we dat GPA goed werd verdragen in een gerandomiseerde, placebo-gecontroleerde studie. De interventies bestonden uit één week dagelijkse orale toediening van GPA in een subtherapeutische dosis

van 100 mg, creatine 5 gram of placebo. Vierentwintig gerandomiseerde deelnemers ontvingen de toegewezen interventie. Er waren geen (ernstige) bijwerkingen met GPA, creatine en placebo na 1 week actieve behandeling. Er werden geen afwijkingen gemeld bij lichamelijk onderzoek, bij toxiciteitstesten in het laboratorium, waaronder nier- en leverparameters, of in cardiovasculaire veiligheid, inclusief QT-interval tussen behandelingsarmen. Dit zijn de eerste menselijke gegevens van de specifieke CK-remmer en potentiële nieuwe bloeddrukverlagende stof GPA. We vonden geen bewijs van toxiciteit met subtherapeutische doses. De volgende stap is om een dosis-escalatie-onderzoek te verrichten om veiligheid en tolerantie in hogere doses bij gezonde vrijwilligers te onderzoeken. Concluderend, hebben we in dit proefschrift de rol van CK in de ontwikkeling van hypertensie onderzocht, mogelijk gebaseerd op de energie die nodig is om hoge bloeddruk te creëren. We hebben laboratorium-, klinische en populatiestudies verricht over CK en hypertensie bij mensen en een nieuw model voor bloeddrukverlaging van CK-remming ontwikkeld.

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AMC graduate school for Medical Sciences PhD portfolio
Summary of PhD training, teaching and parameters of esteem

Name	F.A. (Fares) Karamat
Title PhD	The Role of Creatine Kinase in Hypertension: Therapeutic Perspectives
PhD period	2012 to 2016
Promotores	Prof. dr. S. Middeldorp and Prof. dr. E.T. van Bavel
Co-promotores	Dr. L.M. Brewster
Institution	Department of Vascular Medicine, Amsterdam University Medical Centers Location AMC, Amsterdam, The Netherlands

I	PhD training	Year	Workload (ECTS)
General courses			
	Crash course: Chemistry, biochemistry and molecular biology for MD's (re) entering scientific research	2017	1.0
	Clinical Epidemiology: Systematic Reviews	2016	0.6
	Clinical Epidemiology: Randomized Clinical Trials	2015	0.6
	Practical biostatistics	2013	1.0
	Scientific writing in English for publication	2013	0.5
	The AMC World of Science: fundamental knowledge and skills for scientific research in preparing the PhD thesis	2013	0.7
Seminars, workshops and master classes			
	Two-weekly research meeting Hypertension group	2013-2018	3.0
	Weekly clinical education. Dept. of Vascular Medicine	2011-2013	1.0
	Weekly journal club meeting. Dept. of Vascular Medicine	2011-2013	1.0
	Weekly research meeting Hypertension group. Dept. Vascular Medicine	2011-2013	1.0
	Weekly research meeting Lab. of Molecular Obstetrics Research Group	2011-2013	1.0
(Inter)national scientific presentations			
	Creatine kinase and blood pressure: a systematic review. European Society of Cardiology. Paris, France.	2019	0.5
	Blood Pressure, Hemodynamics and Renal Sodium Excretion in African and European Men on a High Sodium Diet: the Potential Role of CK. 28th European Meeting on Hypertension and Cardiovascular Protection. Barcelona, Spain.	2018	0.5
	The Acute effect of the Creatine kinase inhibitor Beta-GPA in Healthy man (ABC-Trial): a Randomized Placebo Controlled Trial. AHA Council on Hypertension and American Society of Hypertension. San Francisco, California, USA.	2017	0.5
	On the use of Isosorbidedinitrate and Hydralazine for heart failure in patients of African ancestry in the Netherlands. 27th European Meeting on Hypertension and Cardiovascular Protection. Milan, Italy.	2017	0.5

On the use of Isosorbidedinitrate and Hydralazine for heart failure in patients of African ancestry in the Netherlands. European Society of Cardiology. Barcelona, Spain.	2017	0.5
The acute effect of the specific creatine kinase inhibitor beta- gpa in healthy man (abc-trial): a randomized placebo and active controlled first-in-human trial. 26th European Meeting on Hypertension and Cardiovascular Protection. Paris, France.	2016	0.5
Prehypertension and hypertension in urban suriname: the HELISUR study. International Society of hypertension. Seoul, South-Korea.	2016	0.5
Tolerance to the acute effect of the creatine kinase inhibitor beta-guanidinopropionic acid (ABC-Trial): A randomized placebo-controlled first-in-man trial. 25th European Meeting on Hypertension and Cardiovascular Protection. Milan, Italy.	2015	0.5
The acute effect of beta-guanidinopropionic acid in healthy man (ABC Trial) study protocol. Creatine in Health, Sport & Medicine Conference. Laufen, Germany.	2015	0.5
Sodium excretion is associated with creatine kinase activity. International Society of Nephrology & World Congress of Nephrology. Cape Town, South Africa.	2015	0.5
Resistance artery creatine kinase mRNA and blood pressure in humans. Netherlands Hypertension Society. Utrecht, the Netherlands.	2015	0.5
Creatine kinase as a novel therapeutic target for hypertension. Netherlands Hypertension Society. Utrecht, the Netherlands.	2015	0.5
The high creatine kinase syndrome. Surinamedag. The Hague, the Netherlands	2015	0.5
Creatine kinase and the life time cumulative incidence of fainting in the general population. Surinamedag. The Hague, The Netherlands.	2014	0.5
Predictors of peripheral resistance in the general population. 23th European Meeting on Hypertension and Cardiovascular Protection. Milan, Italy.	2013	0.5
Creatine kinase and the life time cumulative incidence of fainting in the general population. 23th European Meeting on Hypertension and Cardiovascular Protection, Milan, Italy.	2013	0.5
Intima and media abnormalities of resistance arteries of hypertensive women. 23th European Meeting on Hypertension and Cardiovascular Protection. Milan, Italy.	2013	0.5
Creatine kinase mRNA expression in resistance arteries and different human tissues. University of Paris-Sud, Faculty of Pharmacy. Paris, France.	2012	0.5
CK-B gene transcriptional activity in human resistance arteries correlates with blood pressure.22th European Meeting on Hypertension and Cardiovascular Protection. London, England.	2012	0.5
International scientific congresses		
European Society of Cardiology. Paris, France.	2019	1.0
28th Scientific meeting European Society of Hypertension and Cardiovascular Protection. Barcelona, Spain	2018	1.0
American Heart Association Council on Hypertension and American Society of Hypertension. San Francisco, California, USA	2017	1.0
European Society of Cardiology. Barcelona, Spain	2017	1.0

	27th Scientific meeting European Society of Hypertension and Cardiovascular Protection. Milan, Italy	2017	1.0
	26th Scientific meeting International Society of Hypertension. Seoul, South Korea.	2017	1.0
	26th Scientific meeting European Society of Hypertension and Cardiovascular Protection. Paris, France	2016	1.0
	European Society of Cardiology. Rome, Italy	2016	1.0
	AMSTOL Symposium. Amsterdam, the Netherlands	2016	1.0
	World Congress of Nephrology and International Society of Nephrology. Cape Town, South Africa	2015	1.0
	25th Scientific meeting European Society of Hypertension and Cardiovascular Protection. Milan, Italy	2015	1.0
	European Society of Cardiology. London, United Kingdom	2015	1.0
	Creatine in Health, Sport & Medicine Conference. Laufen, Germany	2015	1.0
	Netherlands Hypertension Congress. Utrecht, the Netherlands	2015	1.0
	24th Scientific meeting European Society of Hypertension and Cardiovascular Protection. Athens, Greece	2014	1.0
	23th Scientific meeting European Society of Hypertension and Cardiovascular Protection. Milan, Italy	2013	1.0
	22th Scientific meeting European Society of Hypertension and Cardiovascular Protection. London, England	2012	1.0
II	Teaching	Year	Workload (ECTS)
Tutoring, mentoring			
	Shayer Mohan. Bachelor thesis. Blood vessel wall structure in hypertension: A systematic review	2016-2017	1.0
	Marthe Holtmaat, Master thesis. The acute effect of beta-guanidinopropionic acid versus creatine or placebo in healthy man (ABC Trial): a randomised controlled trial	2014-2015	1.0
	Irene Vogel, Master thesis. The correlation between 24-hour blood pressure and sodium excretion	2014-2015	1.0
III	**Parameters of Esteem**	**Year**	
Awards and prizes			
	AFHRE Award for Patient-Oriented or Clinical Research in Hypertension. American Heart Association Council on Hypertension and American Society of Hypertension. San Francisco, California, USA	2017	
	FIGON award from Innovative Drug Research in the Netherlands. Woerden, the Netherlands.	2017	
	Travel grant from the Jaap Schouten Foundation. Rotterdam, the Netherlands	2016	
	Travel grant from Het Amsterdamse Universiteitsfonds, University of Amsterdam. Amsterdam, the Netherlands.	2016	
	Travel grant for young researchers from International Society of Hypertension	2016	

Van Walree grant by The Royal Netherlands Academy of Arts and Sciences (KNAW). Amsterdam, the Netherlands	2015	
Nominated for the Alberto Ferrari prize by European Society of Hypertension and Cardiovascular Protection. Milan, Italy	2015	
Nominated for the Gert van Montfrans Hypertension prize by Netherlands Hypertension Society. Utrecht, the Netherlands	2015	
Travel grant from European Society of Hypertension and Cardiovascular Protection 2015. 25th Scientific meeting of the European Society of Hypertension and Cardiovascular Protection. Milan, Italy	2015	
Travel grant from European Society of Hypertension and Cardiovascular Protection 2013. 23th Scientific meeting of the European Society of Hypertension and Cardiovascular Protection. Milan Italy	2013	
Travel grant from European Society of Hypertension and Cardiovascular Protection 2012. 22th Scientific meeting of the European Society of Hypertension and Cardiovascular Protection. London, England	2012	
Reviewer		
BMJ-Open	2018	1.0
American Journal of Hypertension	2017	1.0
Hypertension Research	2016	1.0
Circulation	2016	1.0
Journal of the American Society of Hypertension	2015	1.0
Journal of Human Hypertension	2013	1.0

Publications

Creatine kinase related publications

1. Brewster LM, Karamat FA, van Montfrans GA. Creatine kinase and blood pressure: a systematic review. Accepted for publication in Medical Sciences.

2. Brewster LM, Oudman I, Nannan Panday RV, Khoyska I, Haan YC, Karamat FA, Clark JF, van Montfrans GA. Creatine kinase and renal sodium excretion in African and European men on a high sodium diet. J Clin Hypertens (Greenwich). 2018 Feb;20(2):334-341. doi: 10.1111/jch.13182.

3. Karamat FA, Horjus DL, Haan YC, van der Woude L, Schaap MC, Oudman I, van Montfrans GA, Nieuwland R, Salomons GS, Clark JF, Brewster LM. The acute effect of beta-guanidinopropionic acid versus creatine or placebo in healthy men (ABC-Trial): A randomized controlled first-in-human trial. Br J Clin Pharmacol. 2017 Dec;83(12):2626-2635. doi: 10.1111/bcp.13390..

4. Karamat FA, Horjus DL, Haan YC, van Montfrans GA, Salomons GS, Clark JF, Brewster LM. The Acute Effect of the Creatine Kinase Inhibitor Beta-GPA in Healthy Man (ABC-Trial): A Randomized Placebo Controlled First-in-human Trial. Hypertension. 2017;70:AP131

5. Karamat FA, Oudman I, Haan YC, van Kuilenburg AB, Leen R, Danser JA, Leijten FP, Ris-Stalpers C, van Montfrans GA, Clark JF, Brewster LM. Creatine kinase inhibition lowers systemic arterial blood pressure in spontaneously hypertensive rats: a randomized controlled trial. J Hypertens. 2016 Dec;34(12):2418-2426.

6. Haan YC, Oudman I, Diemer FS, Karamat FA, van Valkengoed IG, van Montfrans GA, Brewster LM. Creatine kinase as a marker of obesity in a multi-ethnic population. Mol Cell Endocrinol. 2017 Feb 15;442:24-31. doi: 10.1016/j.mce.2016.11.022.

7. Karamat FA, van Montfrans GA, Brewster LM. Creatine synthesis demands the majority of the bioavailable L-arginine. J Hypertens. 2015 Nov;33(11):2368. doi: 10.1097/HJH.0000000000000726

8. Taherzadeh Z, Karamat FA, Ankum WM, Clark JF, van Montfrans GA, van Bavel E, Brewster LM. The Effect of Creatine Kinase Inhibition on Contractile Properties of Human Resistance Arteries. Am J Hypertens. 2016 Feb;29(2):170-7. doi: 10.1093/ajh/hpv078. Epub 2015 Jun 4.

9. Karamat FA, Horjus DL, Haan YC, van der Woude L, Oudman I, van Montfrans GA, Clark JF, Brewster LM. The acute effect of beta-guanidinopropionic acid versus creatine or placebo in healthy men (ABC Trial): study protocol for a randomized controlled trial. Trials. 2015 Feb 22;16:56. doi: 10.1186/s13063-015-0581-9.

10. Karamat FA, Oudman I, Ris-Stalpers C, Afink GB, Keijser R, Clark JF, van Montfrans GA, Brewster LM. Resistance artery creatine kinase mRNA and blood pressure in humans. Hypertension. 2014 Jan;63(1):68-73. doi: 10.1161/HYPERTENSIONAHA.113.01352. Epub 2013 Oct 14.

11. Karamat FA, Clark JF, Brewster LM. Is creatine kinase the intrinsic factor of smooth muscle enhancing vascular contractility in subjects of african ancestry? Hypertension. 2013 Sep;62(3):e7. doi: 10.1161/HYPERTENSIONAHA.113.01853. Epub 2013 Jul 22

12. Karamat FA, van Montfrans GA, Brewster LM. Creatine kinase and pressor response to orthostatic tolerance. Hypertension. 2013 Feb;61(2):e24. doi: 10.1161/HYPERTENSIONAHA.111.00701.

HELISUR-Study project related publications

1. Diemer FS, Baldew SM, Haan YC, Karamat FA, Oehlers GP, van Montfrans GA, van den Born BH, Peters RJG, Nahar-Van Venrooij LMW, Brewster LM. Aortic pulse wave velocity in individuals of Asian and African ancestry: the HELISUR study. J Hum Hypertens. 2018 Dec 19. doi: 10.1038/s41371-018-0144-0. [Epub ahead of print]

2. Diemer FS, Baldew SM, Haan YC, Aartman JQ, Karamat FA, Nahar-van Venrooij LMW, van Montfrans GA, Oehlers GP, Brewster LM. Hypertension and Cardiovascular Risk Profile in a Middle-Income Setting: The HELISUR Study. Am J Hypertens. 2017 Nov 1;30(11):1133-1140.

3. Aartman JQ, Diemer FS, Karamat FA, Bohte E, Baldew SM, Jarbandhan AV, van Montfrans GA, Oehlers GP, Brewster LM. Assessing the feasibility of the Healthy Life in Suriname Study: using advanced hemodynamics to evaluate cardiovascular risk. Rev Panam Salud Publica. 2017 Jun 8;41:e46.

4. Karamat FA, Diemer FS, van Montfrans GA, Oehlers GP, Brewster LM. Arterial stiffness in a random sample of a multi-ethnic population in suriname: the HELISUR study. Journal of Hyperts.2016;34:e132. doi: 10.1097/01.hjh.0000491691.25597.0d

5. Diemer FS, Aartman JQ, Karamat FA, Baldew SM, Jarbandhan AV, van Montfrans GA, Oehlers GP, Brewster LM. Exploring cardiovascular health: the Healthy Life in Suriname (HELISUR) study. A protocol of a cross-sectional study. BMJ Open. 2014;4(12):e006380. Published 2014 Dec 23. doi:10.1136/bmjopen-2014-006380

About the author

Fares Aziz Karamat grew up in Paramaribo, Suriname. After graduating from the Alex Arthur Hoogendoorn Atheneum (Paramaribo, Suriname), he started the study of Medicine at the University of Amsterdam in 2006. During the medical study his interest for science grew with his first research project at the department of Vascular Medicine in 2011. After completing his first scientific internship, which was published in the leading journal "*Hypertension"* as a student researcher, he started a MD/PhD-traject under supervision of Prof. dr. S. Middeldorp and Prof. dr. E.T. van Bavel, which resulted in this thesis. In 2016 he graduated and started his career as medical doctor at the department of Internal Medicine in OLVG-West under supervision of dr. M.C. Weijmer. He is mainly interested in cardiovascular disease and sub-investigator of international lipid lowering clinical trials at the department of Cardiology Amsterdam UMC location VUmc. He aspires a career as an Internist.

"The important thing is not to stop questioning; curiosity has its own reason for existing. Albert Einstein"