BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF CREATINE SUPPLEMENTATION IN RENAL FAILURE

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LIST OF ABBREVIATIONS

Met	Methionine
SAM	S-adenosyl-methionine
SAH	S-adenosyl-homocysteine
Hcy	Homocysteine
THF	Tetrahydrofolate
5,10-CH2 THF	5,10-methylenetetrahydrofolate
5-CH3-THF	5-methyltetrahydrofolate
ADP	adenosine diphospate
AMP	adenosine monophospate
ATP	adenosine triphosphate
ATP-ase:	enzyme, hydrolysing adenosine triphosphate
GAA	guanidinoacetic acid
GAMT	S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (EC 2.1.1.2)
AGAT	L-arginine:glycine amidinotransferase (EC 2.1.4.1)
MHC	Myosin heavy chain
tHcy	total plasma homocysteine

CHAPTER 1

Introduction

Were I to await perfection, my book would never be finished.

Tai T'ung, The Six Scripts: Principles of Chinese Writing

INTRODUCTION

In recent years creatine has been used as a muscular performance enhancing substance in numerous studies. In this thesis we have investigated the effects of creatine administration to animals and patients with renal failure with respect to muscle metabolism and homocysteine metabolism. As an introduction to the original studies, three major topics, reflecting the 3 chapters in the experiments' section will be elaborated. A general overview of creatine metabolism will be presented, together with the metabolic link to the homocysteine metabolism. Safety and analytical aspects are introduced in this section. General background information on muscular energetics and composition is presented, with a rationale for the use of creatine as a performance enhancing substance.

1. Creatine and skeletal muscle energy metabolism

1.1 Synthesis and tissue distribution of creatine

(Wyss et al., 2000; Walker, 1979; Persky et al., 2001)

Creatine is a non-proteogenic nitrogenous substance with a molecular mass of 131 Da, present in high amounts in the human body. A 70-kg person contains about 120 g creatine. About 95% is found in the skeletal muscles. Other creatine containing organs are brain, testes and heart. About 1-2 g/d of creatine is synthesised endogenously and 1-2 g/d is provided exogenously by the diet. Creatine is found mainly in fish and meat products in concentrations between 5-10 g creatine/kg meat. With normal intake, creatine is metabolised to creatinine and subsequently excreted by the kidney. The presence of creatine in the urine is rare and is usually encountered during muscle trauma (e.g. myocardial infarction, myositis) or creatine ingestion (Delanghe et al, 1995). In vegetarians, creatine intake by food is lower due to the absence of meat products in the diet and serum creatine concentrations are lower. (Delanghe et al, 1989).

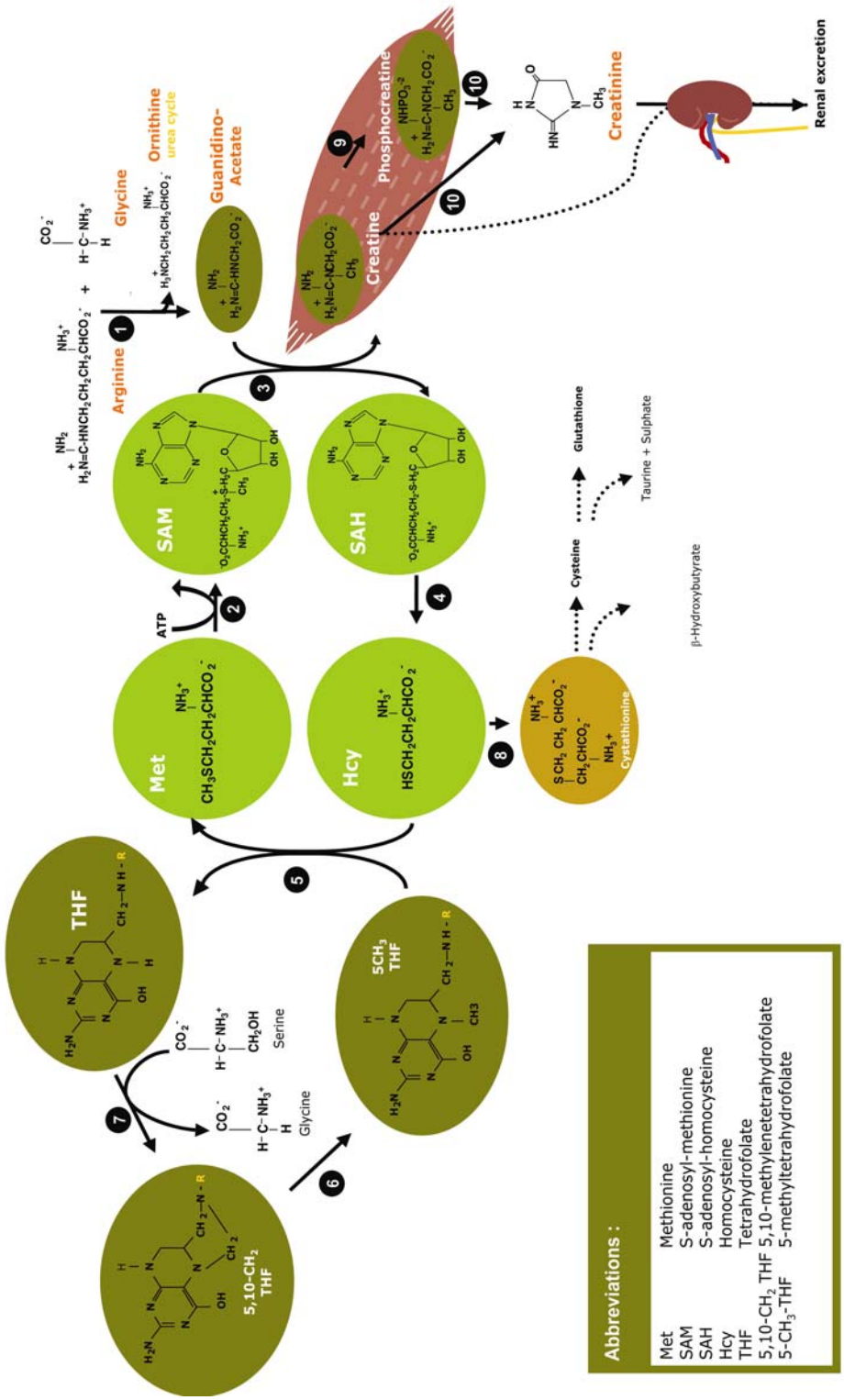
Creatine synthesis *in vivo* (Figure 1.1.) proceeds through two successive metabolic steps. First, guanidinoacetic acid is formed from arginine and glycine, with transfer of the guanidino (amidino) - group from arginine to glycine, catalysed by L-arginine:glycine amidinotransferase (AGAT; EC

2.1.4.1). Creatine is synthesised from guanidinoacetic acid by methylation through methyl donation from S-adenosylmethionine, catalysed by S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2). In contrast to lower vertebrates (fish, birds, amphibiae) which have both the AGAT and GAMT enzyme present in the liver, creatine synthesis in humans exhibits a distinct compartmentalisation. In humans, the kidney exhibits high AGAT activity, whereas it contains almost no GAMT activity. Most of the GAMT activity is situated in the liver. Sites of creatine synthesis and creatine storage are separated. This compartmentalisation implies transport of guanidinoacetic acid and creatine in the bloodstream. Creatine is taken up by tissues with high and fluctuating energy demand, such as striated muscle, heart muscle and spermatozoa, through a specific Na^+-dependent creatine transporter.

Figure 1.1. *(next page)*

Overview of creatine and homocysteine metabolism.

❶ Arginine:glycine amidinotransferase (AGAT)
❷ Methionine adenosyltransferase
❸ Guanidinoacetate methyltransferase (GAMT)
❹ S-Adenosyl homocysteine hydrolase
❺ Methionine synthase
❻ 5,10-CH_2-THF reductase
❼ Serine-hydroxy methyltransferase
❽ Cystathinine-β–synthase
❾ Creatine kinase
❿ Non-enzymatic degradation


Arginine
Glycine
Ornithine
urea cycle
Guanidino-
Acetate
Creatine
Phosphocreatine
Creatinine
Renal excretion
SAM
SAH
Met
Hcy
ATP
THF
5,10-CH2
THF
5CH3
THF
Serine
Glycine
Cysteine
Glutathione
Taurine + Sulphate
β-Hydroxybutyrate
Cystathionine
Abbreviations :
Met Methionine
SAM S-adenosyl-methionine
SAH S-adenosyl-homocysteine
Hcy Homocysteine
THF Tetrahydrofolate
5,10-CH2 THF 5,10-methylenetetrahydrofolate
5-CH3-THF 5-methyltetrahydrofolate

1.2 Skeletal muscle composition and metabolism

In mammals, skeletal muscles are a major source of energy consumption and heat generation with a large variation according to the activity of the skeletal muscles. At rest, overall energy consumption is relatively low. During activation, force generation and active shortening, there is a dramatic increase in energy demand. The mechanisms by which the muscles regulate the energy metabolism in conditions of changing energy demand are complex and remain only partially understood. This section will only briefly address this topic, as background for the presented studies in this thesis. The main focus will be on the myosin motor and associated energy metabolism.

Skeletal muscle is composed of several fiber types that substantially differ in functional and metabolic characteristics (Schiaffino et al., 1996; Pette et al., 1990; Thomason et al., 1986; Pette et al., 2000; Schiaffino et al., 1994). Mammalian skeletal muscle fibers have been characterized histochemically, functionally and biochemically into:

- Red, slow-twitch fibers (type I)
- White, fast-twitch, glycogenolytic (IIx, IIb) fibers and oxidative (IIa) fibers

The terminology (Pette et al., 1999; Schiaffino et al., 1994), referring to the different types of muscle fibers is based on different properties of the muscle fibers, using different methods: (1) From a functional point of view, the terms slow- and fast-twitch have been attributed to the muscle fibers according to the contraction speed during unfused tetani, and fatigue properties during fused tetani. (2) Histochemically fiber typing is based on the different pH-sensitivity of the myofibril ATP-ase activity in fibers of slow- and fast-twitch muscle (Barrany, 1967). (3) Biochemically, slow-twitch fibers contain high concentrations of myoglobin and thus appear red on inspection, together with a high activity of oxidative enzymes. The fast-twitch muscles are called 'white' due to a lower myoglobin concentration and are mainly glycogenolytic in their energy metabolism. (4) Molecularly, the ATP-ase activity of the muscle fibers, and thus contractile characteristics, depend mainly on the myofibrillar protein composition and especially the myosin heavy chain isoforms. Based on the expression of the myosin isoforms (I, IIa, IIx, IIb in rats; I, IIa and IIx in humans), different metabolic activity and contractile characteristics are observed in skeletal muscles.

Type I fibers are well adapted for aerobic energy production. These fibers exhibit a low myosin ATP-ase activity but have a high mitochondrial

density, high oxidative enzyme activity profile, high cytochrome c concentration and rich capillary supply. Type II fibers are subdivided in IIa, IIx and IIb-fibers, with increasing myosin ATP-ase activity levels. The type II fibers are suited to anaerobic energy production and are characterised by a low mitochondrial density, high glycolytic enzyme activity profile, high creatine kinase activity, low myoglobin concentration, poor capillary supply and a high myosin ATP-ase activity. Due to the difference in myoglobin content between fiber types, muscles composed of predominantly type I fibers are designated as red muscles, whereas muscles predominantely composed of type II fibers are referred to as white muscles (Schiaffino et al., 1996). The expression of the different MHC-isoforms is not fixed and can change under several conditions. Inactivity, metabolic factors,… influence the expression of the MHC-gene in the skeletal muscle and in conditions of disuse, a general slow-to-fast transition is observed (Baldwin et al., 2001; Schiaffino et al., 1996; Pette et al., 1990).

1.3 Uraemic myopathy

Patients with chronic renal failure experience muscle dysfunction for a variety of reasons (Ritz et al., 2001; Clyne et al., 1993; Campistol, 2002). The myopathy associated with renal failure can be a manifestation of the underlying disease, neuropathy or aggravated by drug toxicity. It is characterised by an increased protein catabolism, mainly due to a higher ubiquitin-proteasome activity (Mitch et al., 2002). Acidosis, hyperparathyroidism, inactivity and ischemia can influence this myopathy. A vitamin D-responsive myopathy is often found in patients with renal failure, though the aetiology is not fully understood and probably multifactorial.

1.4 Role of creatine in muscle energy metabolism

The intramuscular creatine concentrations range from 110 to 160 mmol/kg dry weight in humans, whereas the creatine concentration in animals can vary between species (Edström et al., 1982). In skeletal muscle, about 60% of the creatine is present in its phosphorylated form: phosphocreatine. Phosphocreatine plays a pivotal role in the energetic balance during muscle contraction, especially during high-intensity exercise. Energy for muscle contraction and relaxation is uniquely provided by the dephosphorylation of ATP. The sustainability of muscle contraction depends on the availability of ATP. The ATP-concentration in the skeletal muscle is about 24 mmol/kg dry weight. Without energy replenishing systems, ATP would be depleted within 2 seconds after onset of contraction (Figure 1.2.). During the first seconds of muscular contraction, ATP is replenished by the anaerobic degradation of phosphocreatine and glycogen (Maughan, 1999).

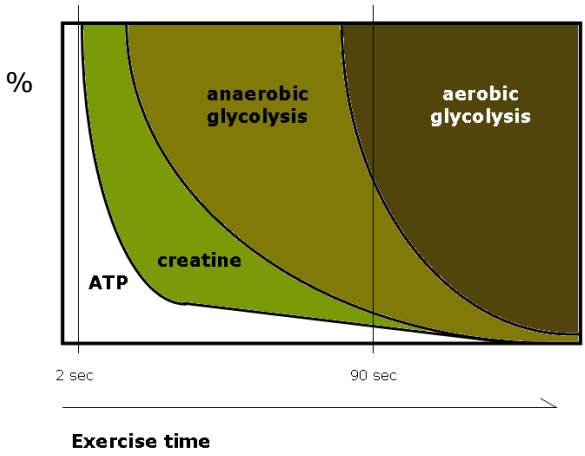


Figure 1.2. *Proportional contribution of the different energy systems during exercise of different duration*

These ATP-regenerating systems can maintain muscular ATP-concentrations, sufficient for muscular contraction during both a single bout of exercise and repeated bouts of high-intensity exercise. This temporal energy buffer system of the creatine-phosphocreatine system is the best known function of creatine.

A second function of the creatine-phosphocreatine system consists of a cellular energy transport system (creatine-phosphocreatine shuttle). Older scientific insights state that intracellular energy translocation is entirely dependent on the diffusion of ATP and ADP from sites of energy production to sites of energy utilisation and vice versa (Meyer et al., 1984). In 1966, the concept of the creatine-phosphocreatine shuttle was introduced (Bessman et al., 1966). This provided insight in the movement of cellular chemical energy between several compartments in the cell. In this model (Wallimann et al., 1992; Saks et al., 1996), creatine and phosphocreatine are considered as cellular energy carriers and this model is supported by the existence of different isoforms of creatine kinase in discrete cellular locations with different metabolic roles. As the outer mitochondrial membrane is relatively impermeable to adenine nucleotides, creatine and phosphocreatine are more plausible energy carriers. In this regard, the creatine-phosphocreatine system is supportive for the 'classical' ATP-ADP system. The relative importance of each of these systems to cellular spatial energy transfer remains a matter of debate.

A third function of creatine-phosphocreatine is a low threshold ADP-sensor. This sensor maintains the ATP/ADP ratios in subcellular locations. In the mitochondrion, creatine can react with ATP, derived from mitochondrial respiration in a reaction catalysed by mitochondrial creatine kinase, increasing the local ADP-concentration and stimulating the mitochondrial respiration. In this regard, Walsh et al. demonstrated in human skeletal muscle that by addition of creatine to a mitochondrial incubation medium,

ADP-stimulated mitochondrial respiration was increased (Walsh et al., 2001). When phosphocreatine was added to the medium however, ADP-stimulated mitochondrial respiration was reduced, and the effect was greater than the opposite effect of creatine on respiration. Phosphocreatine is likely to be a more potent regulator of mitochondrial respiration than creatine. Cellular phoshocreatine/creatine ratios can direct mitochondrial respiration and the creatine-phosphocreatine system functions as a low threshold ADP-sensor which functionally couples ATP-consuming and -producing pathways.
During ATP-synthesis from phosphocreatine, protons are consumed and provide a pH-buffer against lactic acid formation during exercise (Sahlin, 1978).

$$\textbf{Phosphocreatine + ADP + } H^{+} \xrightleftharpoons{\text{Creatine kinase}} \textbf{ATP + Creatine}$$

1.5 Creatine supplementation

As described in the previous paragraph, creatine plays a central role in the energy metabolism of the muscle cell, especially during the first phase of muscle contraction (Figure 1.2.). Hence the hypothesis was developed that muscular performance could be enhanced by increasing the cellular concentrations of creatine and phosphocreatine (Casey et al., 2000; Greenhaff et al., 1994; Francaux et al., 2000).

Theoretically creatine can have several metabolic interactions and modes of action:
(1) Maintaining a high ATP/ADP ratio during exercise.
(2) Buffering of protons, accumulating in the acidic environment of anaerobic exercise.
(3) Enhancing the creatine/phosphocreatine energy shuttle with a more efficient transfer of energy to sites of ATP use.
(4) Stimulating protein synthesis in the muscle.

Short-term creatine supplementation with increased intramuscular creatine stores was first described by Harris (Harris et al., 1992). Muscular creatine stores can increase 10-20% after ingestion of 20 g/d creatine during 5 days. Following creatine supplementation, intramuscular creatine concentration seem to have a maximum of about 160 mmol/kg dry weight and the increase in muscular creatine depends on the initial creatine concentration (Harris et al., 1992; Greenhaff et al., 1994). Prolonged intake of creatine was found to elevate intramuscular creatine concentrations using lower dosages (Hultman et al., 1996; Vandenberghe et al., 1997). Creatine loading in muscle was found to increase when creatine was ingested with carbohydrate (Green et al., 1996). Creatine was

also found to increase muscle glycogen concentrations (Robinson et al., 1999; Op 't Eijnde (1) et al., 2001; Van Loon et al., 2004) and GLUT4-expression (Op 't Eijnde (2) et al., 2001; Derave et al., 2003; Ju et al., 2005).
The effect of creatine on muscle protein synthesis is less studied. Early studies suggested an important role for creatine in promoting muscular protein synthesis (Ingwall, 1974; Ingwall 1974). Studies on muscular protein synthesis and breakdown in human volunteers could not demonstrate a beneficial effect of creatine on protein synthesis or breakdown (Louis et al (1), 2003; Louis (2), 2003).

Countless studies in humans have examined the effects of creatine supplementation on exercise performance (Kreider et al., 1998; Kraemer et al., 1999; Engelhardt et al.,1998; Demant et al., 1999). Several reviews summarising the ergogenic effects of creatine have been published (Terjung et al., 2000; Williams et al., 1998; Sheppard et al., 2000; Bemben et al., 2005). Earlier studies focussed on exercise performance using laboratory tests (Greenhaff et al., 1993; Leemputte et al., 1999; Maganaris et al., 1998; Kreis et al., 1998, whereas more recent studies focus on the effects of creatine on sport-specific performance on field tests (Rossiter et al., 1996; Preen et al., 2001; Peyrebrune et al., 1998; Chrusch et al., 2001). In controlled laboratory tests, creatine was found to enhance exercise performance of short-term, high intensity exercise. The effect is even more pronounced in schedules with repeated bouts of exercise.
After immobilisation, creatine was found to enhance muscular rehabilition by changes in MRF4 and myogenin expression and therefore promoting muscular hypertrophy (Hespel et al., 2001).
There is less convincing evidence for the performance enhancing effects of creatine during endurance exercise. The creatine-phosphocreatine system has only a limited role in exercise tasks of 1.5-3 min. Therefore creatine supplementation may not prove ergogenic in exercises of longer duration. The effects of creatine on field tests are less consistent. Most of the evidence on the beneficial effect of creatine on muscular performance derives from short-term studies in healthy athletes, whereas the long-term effect of creatine in animal models of neuromuscular disorders and in patients with these disorders remains controversial (Derave et al., 2003).
Considering the possible health risks of creatine supplementation, several aspects can be discussed. There are only anecdotal reports on side effects of creatine supplementation in humans (e.g., muscle cramping, muscle injury, thermoregulation and renal complications). The only side-effect, consistently reported in literature is an acute (over 3–6 days) gain in body mass of 0.5-2 kg. This gain in body weight appears to be primarily due to increased muscle water storage (Häussinger et al., 1993; Ziegenfuss et al., 1998). Robinson (Robinson, 2000) described a case of a weight-lifter who developed an acute quadriceps compartment syndrome with

rhabdomyolysis and renal failure after high-dose creatine ingestion. Mendel (Mendel et al., 2005) investigated the effect of creatine on thermoregulatory responses while exercising and found no negative effects. With short-term supplementation (days to weeks), other adverse effects appear to be anecdotal and non-consistent or minimal (Benzi, 2000; Graham et al., 1999; Poortmans et al., 1997; Poortmans et al., 1999; Schilling et al., 2001; Volek et al., 2001). Safety of prolonged (months to years) use of creatine remains undetermined as only limited data are available. The absence of adverse reports with prolonged creatine use does not guarantee that chronic use is without risk. In young healthy adults, creatine is generally considered as safe (Pline et al., 2005; Groeneveld et al., 2005), though in elderly and renal disease patients, only limited data is available. Though controlled studies failed to demonstrate major negative effects of creatine, concern is still warranted when evaluating the impact of creatine ingestion on the renal function and possible carcinogenic potential. Preliminary data in our lab (Taes et al., unpublished data) demonstrated altered global DNA-methylation patterns in creatine treated animals. In both control animals and animals with renal failure, creatine supplementation was associated with lower (21%, p=0.03; (unpublished data, Y. Taes)) hepatic 5-methylcytosine concentrations. This finding contrasts with the decreased tHcy-concentrations and the increase in folic acid concentrations observed in this animal model. These findings, let us hypothesise that creatine could increase DNA-methylation by desinhibiting transmethylation of DNA through lower Hcy-concentrations. However we have observed lower DNA-methylation in association with creatine supplementation. A direct effect of creatine on DNA could explain these paradoxal findings. However low DNA-methylation is described to be associated with carcinogenesis and diseases with imprinting disorders (Paulsen et al., 2005). The effects of creatine on DNA-methylation and carcinogenetic risk needs further research.

Analytical aspects of creatine and creatinine determination

Apart from the *in vivo* degradation of creatine to creatinine, creatine could also interfere analytically in the determination of serum creatinine. Serum creatinine is routinely measured colorimetrically using the Jaffé's reaction. Originally described in 1886 this reaction suffers from interference of bilirubin and non-specific chromogens such as ketones… and has been modified extensively over the decades. Today, several assays exist with varying propensity to interference of non-specific chromogens. Rate blanked kinetic assays suffer less from non-specific interference then end-point assays. However with the use of random access analysers, the deproteinisation step was omitted in the analysis and the protein error is substantial in this kind of assay. Creatine can interfere with the Jaffé

reaction as a non-specific chromogen with higher apparent creatinine concentrations (Taes et al., 2005)

2. Creatine, methylation demand and homocysteine metabolism

As described previously in figure 1.1., creatine and homocysteine metabolism are metabolically linked (Stead et al., 2001; Steenge et al., 2001). In this paragraph, homocysteine and folic acid metabolism will be briefly summarised in relation to creatine metabolism.

Homocysteine (Hcy) (Selhub, 1999; Hoffer, 2004) is a sulphur-containing amino acid, formed by demethylation of methionine and plays an important role in the activated methyl and folate cycle (figure 1.1.). Methionine is converted intracellular to S-adenosylmethionine, a sulphonium compound with a highly reactive methyl group, which acts as a universal methyl donor in numerous transmethylation reactions *in vivo*. The demethylated product S-adenosyl homocysteine, a thioether, is readily hydrolyzed to Hcy and adenosine by S-adenosyl homocysteine hydrolase.
Elevated concentrations of plasma Hcy (total plasma Hcy (tHcy) are described in several populations to be associated with an increased cardiovascular mortality and morbidity (Refsum et al., 1998; Homocysteine Studies Collaboration, 2002; Wald et al., 2002; Ridker et al., 2002). In renal failure patients, Hcy is thought to contribute to the high prevalence of cardiovascular disease (Selhub, 1999; Moat et al., 2004).
Hyperhomocysteinemia can be caused by: (1) mutations of the genes encoding for key enzymes in the homocysteine metabolism, such as the Cystathionine β-synthase deficiency. Homozygosity for the methylenetetrahydrofolate reductase 677C[PI]T transition, leading to the unstable 'thermolabile form' of this enzyme, is particularly frequent and is responsible for mild hyperhomocysteinemia and increased cardiovascular risk (Klerk et al., 2002); (2) reduced intake or intestinal absorption of folic acid, vitamin B12, or vitamin B6 (rare); (3) the administration of certain drugs (L-DOPA, methotrexate, etc.) and (4) chronic renal failure. The mechanism of hyperhomocysteinemia in renal failure remains incompletely understood, but both a decreased renal metabolism of Hcy (Refsum et al., 1985; Guttormsen et al., 1997; Van Guldener et al., 1998; Van Guldener et al., 1999) and an inhibition of the Hcy-metabolising enzymes by the uraemic environment are thought to play a role (Perna et al., 2003; Perna et al., 2001).

Treatment of hyperhomocysteinemia mainly consists of alimentary folic acid and vitamin B6 and B12 supplements. An average reduction of about 30% in tHcy is observed in uraemic patients treated with folic acid (De

Vriese et al., 2002). The effect of vitamin B6 and B12 is in general much less pronounced (Bostom et al., 1997). Supplementation with other methyl-donating molecules such as betaine can decrease post-methionine loading tHcy-concentrations by 18%, in patients already treated with folic acid supplementation (McGregor et al., 2002; Wald et al., 2001; Splaver et al., 2004). However, even combined high-dose folic acid and vitamin B6 or B12 supplementation cannot entirely normalize tHcy in renal failure patients.

The importance of hyperhomocysteinemia in determining cardiovascular risk remains unknown. Though *in vivo* and *in vitro* evidence points out towards a role for homocysteine in atherogenesis by endothelial injury, platelet activation, smooth muscle proliferation, oxidation of lipoproteins and leukocyte-endothelial interactions (Thambyrajah (1) et al., 2000), not all epidemiological evidence supports a role for tHcy in the cardiovascular risk. Moreover, tHcy-lowering intervention trials with folic acid or multivitamin preparations demonstrate lower tHcy-concentrations, but fail to demonstrate a reduced cardiovascular risk with lower tHcy-concentrations. Homocysteine lowering by folic acid treatment (Van Guldener et al., 2000; Thambyrajah (2) et al., 2000) failed to show a beneficial effect on endothelial function or large artery stiffness in uraemia, while others report decreased markers of lipid peroxidation with folic acid treatment but no association of tHcy-concentrations and cause of death (Bayes et al., 2001, Bayes et al., 2003). The relation tHcy-cardiovascular risk is still unclear and a 'reverse epidemiology' has been described in uraemic patients with higher cardiovascular risk associated with lower tHcy-concentrations (Kalantar-Zadeh et al., 2004; Suliman et al., 2005) in contrast to the positive association in the general population. Malnutrition, wasting, inflammation and underlying diseases are confounding factors in the relation cardiovascular risk and tHcy-concentrations in uraemia (Suliman et al., 2005).

Creatine metabolism is linked to the Hcy-metabolism by the transmethylation of guanidinoacetic acid to creatine with formation of S-adenosylhomocysteine as a side-product and is further hydrolysed to Hcy (Figure 1.1.). Quantitatively, methylation of guanidinoacetic acid accounts for the majority of the transmethylation reactions in the body (Mudd et al., 1975; Mudd et al., 1980). In this thesis we have investigated the potential use of creatine as a Hcy-lowering compound in both uraemic animals and dialysis patients. The hypothesis behind these studies is that supraphysiological creatine administration can decrease Hcy-formation by lowering the methylation demand through inhibition of endogenous creatine synthesis.

3. Remnant kidney model

3.1 General aspects

Several models of renal failure in animals exist (Gagnon et al., 1983; Esposito et al., 1999; Terzi et al., 2000, Kren et al., 1999; O'Bryan et al., 2000). The models can be divided into induced models and genetic models. In the induced models, renal failure is generally established by destruction of kidney tissue. This can be accomplished by (partial) nephrectomy by ligation of (several branches of) the renal artery, electrocoagulation or thermal ablation of kidney tissue or the administration of nephrotoxins, such as adriamycin or puromycin. The genetic models consist of animal models with spontaneous evolution to renal failure such as the Jck-mice (juvenile cystic kidney), polycystic kidney rats (Han: SPRD-cy), ICGN-mice (spontaneous nephrotic mice with idiopathic crescentic glomerulonephritis). Transgenic animal models with renal failure, such as the Tg26-mouse (oligosyndactyly mice with evolution to proteinuria, renal failure and focal segmental glomerulosclerosis) are classed within the genetic models of renal failure. All these models have different characteristics and large differences in experimental results can be obtained using different animal strains (Esposito et al., 1999).
In this thesis we have used the remnant kidney model to study the effects of creatine supplementation on renal function, skeletal muscle and homocysteine metabolism. The remnant kidney model is an induced model of renal insufficiency by reduction of the renal mass, based on a unilateral nephrectomy and partial infarction of the contralateral kidney. The surgical method for inducing renal failure allows application in several animal species. The technique is moderately easy to perform and the model has been applied mainly in various strains of rats, but also in mice, cats, dogs, rabbits and baboons. Reduced glomerular filtration, glomerular sclerosis and proteinuria are the cardinal features of this model.

3.2 Procedure

In this thesis we have used a two-stage model with cryoablation of the upper and lower pole of the left kidney, followed one week later by a right nephrectomy. The general procedure is described in figures 1.3. and 1.4.

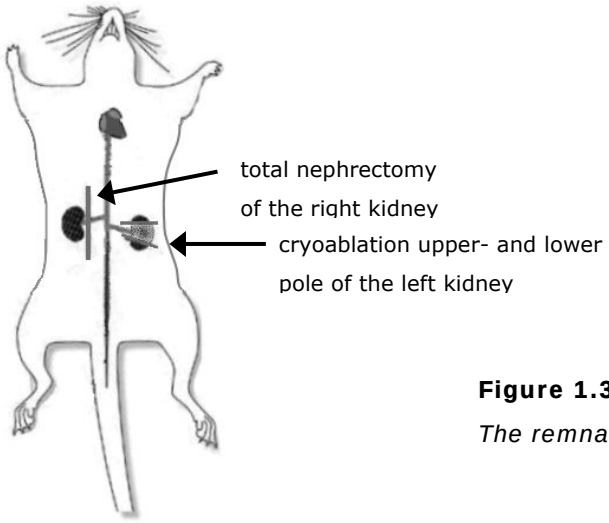
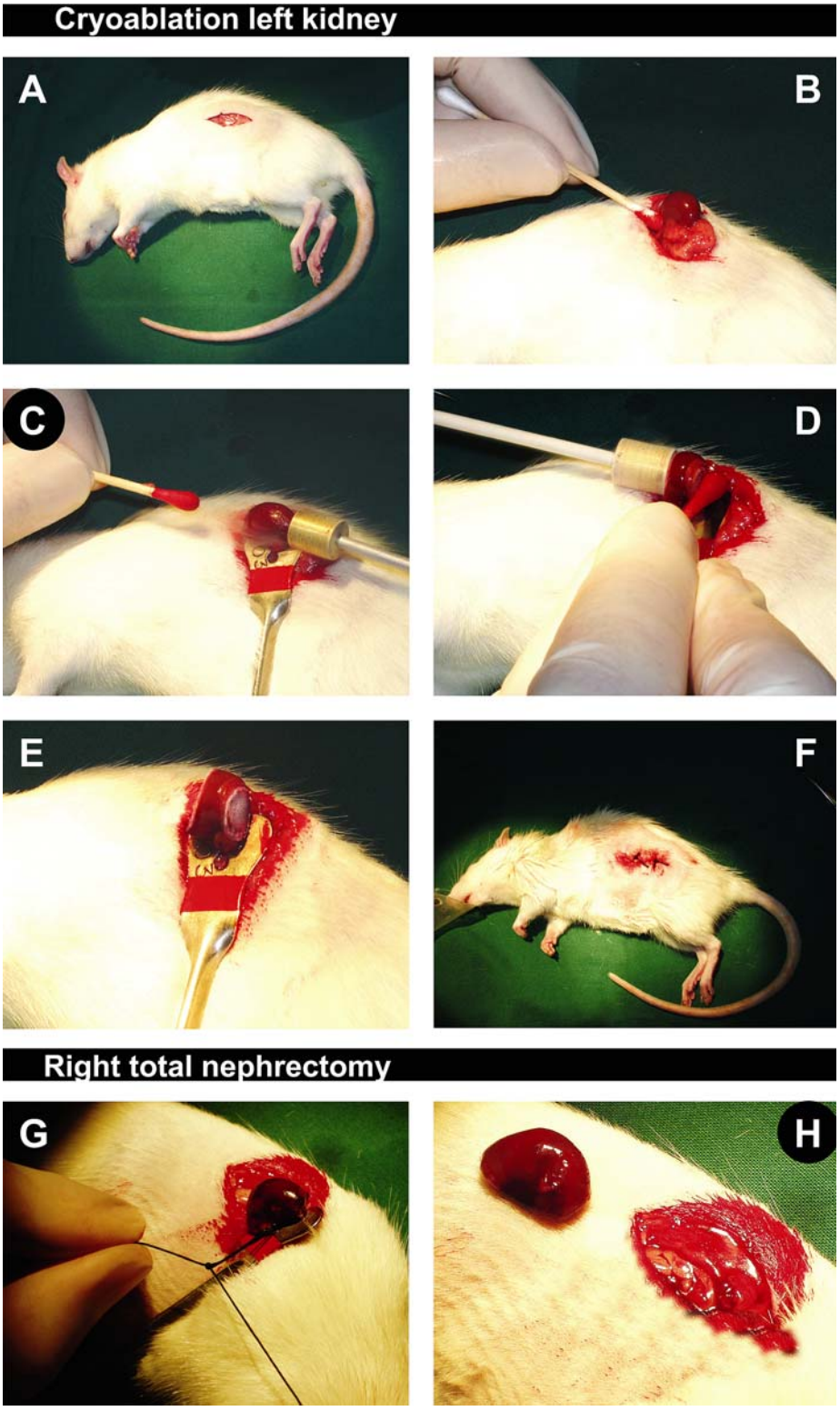


Figure 1.3.
The remnant kidney model in rats.

The animal is anesthesised by inhalation of halothane (Fluothane, Astra-Zeneca). The left kidney is exposed by a lumbar incision (Fig 1.4. **A**, **B**). The upper and lower pole of the left kidney are ablated by application of a copper bar, cooled in liquid nitrogen (Fig 1.4. **C**, **D**).
Figure 1.4. **E** shows the status of the left kidney immediately after cryoablation. The muscles and skin are closed in layers and the animal is allowed to recover (figure 1.4. **F**). During 48h post-operatively, analgesia is provided by administration of buprenorphine IM (Temgesic, Schering-Plough). One week later, using the same surgical approach, the right kidney is removed. The kidney is exposed, trimmed of fat and connective tissue, the vasculature tied (figure 1.4. **G**) and the kidney removed (figure 1.4. **H**). This procedure is used throughout the different experimental chapters for inducing renal failure in rats.

Figure 1.4. *(next page)*
The remnant kidney model in rats, general procedure.


Cryoablation left kidney
A
B
C
D
E
F
Right total nephrectomy
G
H

Analytical and biochemical aspects associated with supraphysiological creatine intake

Letter to the editor

Taes YEC, De Vriese AS
Clin Chim Acta 2005; 351: 217-219

During the past decade, creatine has established itself as an ergogenic aid in sports and has gained increasing attention in medical and lay press as apossible therapeutic agent for various neuromuscular diseases. The available literature mainly covers the short-term effects of creatine supplementation on skeletal muscle function in health and disease [1], while little is known about the efficiency of long-term supplementation and possible side effects. This letter addresses briefly the analytical interference of creatine supplementation on common laboratory tests and examines possible side effects and health risks.
Creatine is linked to the arginine, guanidino-compound, one-carbon and homocysteine metabolism, as well as muscular energy metabolism.

1. Creatine ingestion and renal function parameters

Increased intake of creatine can interfere with the determination of creatinine concentrations. First, the increased creatine load is converted to creatinine and increases the daily excretion of creatinine. Secondly, analytical interference of creatine on the different creatinine assays can increase creatinine concentrations due to the aspecificity of the assay. In this regard, the apparent increases in plasma creatinine after creatine administration can be attributed to the in vivo degradation of creatine to creatinine, but also to analytical interference of creatine with the plasma creatinine determinations. Depending on the assay used, creatine can substantially interfere with routine creatinine determination. In the colorimetric Jaffe´ reaction, creatine can act as pseudochromogen resulting in higher apparent creatinine concentrations. Enzymatic assays, using creatinine-to-creatine conversion in their reaction, are also prone to analytical interference with less predictable deviation from the true creatinine concentration. Using an uncompensated rate-blanked kinetic Jaffe´ assay [2], we investigated the in vitro interference of creatine on this routine creatinine assay. Serum was spiked with increasing concentrations of creatine (0–10 mg/dl). Fig. 1 illustrates the serum creatinine concentrations in function of the creatine concentration added. Creatine addition significantly increased serum creatinine concentrations between 2.5 and 10 mg/dl ($p<0.05$). Endpoint Jaffé-based methods without rate-blanked kinetic correction are prone to larger deviations from the true serum creatinine concentration. In animals with renal failure, serum creatinine concentrations were apparently found to increase upon creatine administration without evidence of deterioration of renal failure, whereas in animals with normal renal function similar creatinine concentrations were observed upon creatine feeding [2].
The safety of high-dose creatine supplementation has been questioned. Two case reports of deleterious effects of creatine on kidney function have been published. The first describes an interstitial nephritis in a previously

healthy young man after ingestion of creatine [3]. In the second, deterioration of renal function in a young man with underlying focal segmental glomerulosclerosis was observed [4]. Although renal function recovered after cessation of creatine, no causal relationship between creatine ingestion and renal failure can be established on the basis of these case reports. In humans, controlled studies did not reveal adverse effects of creatine supplementation. Poortmans et al. [5–7] investigated intensely renal function in young healthy subjects. No alterations in creatinine, urea or albumin clearance were observed during short-term (5 days) or long-term supplementation (1 month to 5 years) with dosages from 1 to 80 g daily. Robinson [8] and Volek [9] reported only transient increases in apparent serum creatinine during creatine ingestion, without evidence of renal dysfunction. Six weeks after cessation of the creatine supplementation, serum creatinine had returned to baseline [8]. In contrast to humans, experiments in animals suggest progression of renal disease in an animal model of human polycystic kidney disease [10]. In a surgical partial nephrectomy animal model of chronic renal failure, no changes in inulin- or creatinine clearance rates nor in renal protein handling were observed [2]. No biochemical evidence is currently available to assume negative effects of creatine supplementation on liver- or gastroenterological functions. A possible health risk of creatine, which is insufficiently studied, is the formation of mutagenic compounds. Creatine and creatinine are described as precursors for amino-imidazo azaarenes, a class of food mutagens, found in meat and fish. By heating the meat, creatine can be converted to these mutagenic and carcinogenic compounds. Whether or not high-dose creatine administration is associated with formation of these compounds in vivo is currently unknown [11]. Creatine is classed as a nutritional supplement and is freely available over the counter. These different formulations are not subjected to adequate quality control as with pharmaceutical preparations. Commercially, creatine is synthesized from cyanamide and sarcosine. Toxic side products or contaminants such as dihydrotriazine and dicyanamide could be present in these formulations. As creatine is ingested in high doses, these contaminants could be ingested in sufficient amount to exert toxic side effects. Moreover, both amateur and professional sportsmen tend to use even higher doses then recommended (>20 g).

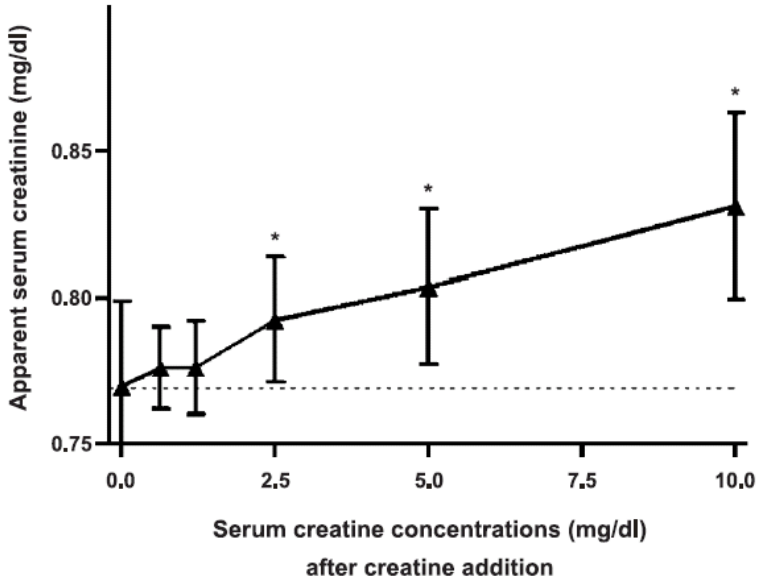


Fig. 1. Apparent serum creatinine concentrations after addition of creatine. * $p<0.05$; serum creatinine concentrations after addition vs. baseline concentrations.

2. Methylation demand and homocysteine metabolism

A more appealing aspect of creatine supplementation in uraemia is the ability to diminish the in vivo methylation demand. Dietary intake (1 g) and endogenous synthesis (1 g) in liver compensate for a daily loss as creatinine of about 2 g. In humans, creatine is synthesized through two successive metabolic steps. Guanidinoacetate is formed in the kidney from glycine and arginine by arginine:glycine aminidinotransferase.

Secondly, guanidinoacetate is methylated in the mammalian liver to creatine by guanidinoacetate-methyltransferase with S-adenosylmethionine acting as methyl-donor. Creatine is transported to the muscle in the blood as free molecule and is actively transported into the muscle cell by a specific transporter [11,12]. Methylation of guanidinoacetate accounts for up to 70% of the transmethylation reactions in the body.

Quantitatively, this exceeds all other methylation reactions. Stead et al. [12] demonstrated that downregulation of endogenous creatine synthesis by creatine supplementation could decrease methylation demand and homocysteine in animals with normal renal function. In animals with renal failure, creatine supplementation was associated with lower homocysteine concentrations and increased folic acid concentrations, despite similar

intakes [13]. However, in humans, a placebo-controlled, cross-over study could not demonstrate any homocysteine lowering potential of creatine supplementation, despite documented uptake of creatine [14]. The effect of creatine on labile methyl pool consumption seems therefore limited in humans.

3. Conclusions

Although creatine is widely used as a performance enhancing nutritional supplement, safety of creatine administration still remains open for discussion. No major effects on renal function have been described in humans; however, side effects of long-term administration are unknown. Analytical interference of creatine ingestion on creatinine determinations is described and should be taken into account when evaluating renal function. Creatine lowers homocysteine concentrations in animals with normal and diminished renal function, though this effect is not observed in humans. The hype around creatine supplementation is decreasing over the years due to several negative studies on creatine supplementation in various diseases.

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CHAPTER 2

Aims and outline of the studies

What lies still is easy to grasp;
What lies far off is easy to anticipate;
What is brittle is easy to shatter;
What is small is easy to disperse.

Yet a tree broader than a man can embrace, is born of a tiny shoot;
A dam greater than a river can overflow starts with a clod of earth;
A journey of a thousand miles begins at the spot under one's feet.

Therefore deal with things before they happen;
Create order before there is confusion.

Tao Te King 64a/81
Lao Tse

AIMS AND OUTLINE OF THE STUDIES

The general objective of this thesis is to investigate the influence of creatine supplementation on biochemical and contractile properties of the striated muscle and the influence on homocysteine metabolism in an animal model of uraemia and in patients. We hypothesised that creatine could beneficially act on muscular performance in renal failure patients as in young healthy subjects. Moreover creatine could lower plasma tHcy-concentrations by lowering endogenous creatine synthesis and methylation demand.

More specific aims can be defined as

1. To examine the renal safety of prolonged creatine supplementation in our animal model of chronic uraemia and the effects on renal function in rats with pre-existing renal failure.
   (chapter 3.1)
2. To characterize the effect of creatine supplementation on plasma and liver concentrations of homocysteine, folate and vitamin B12 in rats with normal and diminished kidney function.
   (chapter 3.2a)
3. To characterize the effect of creatine supplementation on plasma homocysteine, folate and vitamin B12 concentrations in dialysis patients with hyperhomocysteinemia, already treated with folic acid, vitamin B12 and vitamin B6.
   (chapter 3.2b)
4. To investigate the effect of creatine supplementation, together with exercise training on physical performance in aging men.
   (chapter 3.3a)
5. To examine the effect of creatine on myosin heavy chain expression in the rat striated muscle in animals with normal and diminished kidney function.
   (chapter 3.3b)

The series of experiments addressing the research questions are presented in this thesis in the form of 6 manuscripts, published in peer-reviewed journals in the field of nephrology, biochemistry and physiology. The studies encompass both animal and human experiments and are presented in three chapters.

CHAPTER 3

Experiments

Great perfection seems incomplete,
But does not decay;
Great abundance seems empty,
But does not fail.

Great truth seems contradictory;
Great cleverness seems stupid;
Great eloquence seems awkward.

As spring overcomes the cold,
And autumn overcomes the heat,
So calm and quiet overcome the world.
Tao Te King 45/81
Lao Tse

CHAPTER 3.1

Safety of creatine supplementation in an animal model of moderate chronic uraemia

Creatine supplementation does not affect kidney function in an animal model with pre-existing renal failure

Taes YE, et al. Nephrol Dial Transplant 2003;18: 258-264

Abstract

Background. Creatine is widely used as an ergogenic substance among athletes. Safety of prolonged creatine intake has been questioned, based upon case reports and animal data. We investigated the effect of prolonged creatine ingestion on renal function in animals with normal kidney function or pre-existing kidney failure, respectively.

Methods. Male Wistar rats were randomly allocated to four experimental groups: (i) sham-operated, control diet; (ii) sham-operated, creatine-supplemented diet (2% w/w (0.9±0.2 g creatine/kg body weight/day)); (iii) two-thirds nephrectomized, control diet; and (iv) two-thirds nephrectomized, creatine supplemented diet. Glomerular filtration rate was determined using inulin and creatinine clearance, together with albumin excretion, urea clearance, muscle and serum creatine and serum cystatin C concentrations.

Results. In contrast to previous reports, no detrimental effects of creatine supplementation on the renal function indices were observed in two-thirds nephrectomized or sham-operated animals. No differences were observed in inulin (0.28±0.08 vs 0.25±0.08 ml/min/100 g; P=NS) or creatinine clearance rates. Serum cystatin C concentration, urinary protein excretion, and albumin and urea clearance were comparable between creatine-supplemented and control-diet fed animals in both sham-operated and two-thirds nephrectomized animals. Serum creatine and intramuscular total creatine concentrations were higher in creatine-supplemented groups ($P<0.05$).

Conclusions. Creatine supplementation at a dosage of 2% w/w for 4 weeks does not impair kidney function in animals with pre-existing renal failure or in control animals.

Keywords: creatine supplementation; kidney function

Introduction

In recent years creatine has received considerable attention in the medical and lay press and is widely used as an ergogenic substance. Numerous publications have reported the beneficial effect of creatine supplementation on exercise performance, in healthy subjects or animals as well as in patients or animal models of neurodegenerative diseases [1–3].

As early as 1926, Chanutin [4] reported increased creatinine excretion after creatine ingestion in humans. Several studies have described the non-enzymatic degradation of creatine to creatinine, without evidence of altering kidney function. Phosphocreatine is converted to creatinine at a rate of 2.6%/day and creatine at 1.1%/day in vivo [2,4]. Based upon two case reports, safety of prolonged high-dose creatine intake has recently been questioned [5–7], but no causal relationship between creatine ingestion and renal failure can be attributed on the basis of these case reports. Controlled studies [5,8–11] could not demonstrate major adverse effects or health risks of creatine supplementation in humans. Muscle cramps, stomach upset or diarrhoea were reported as sporadic complaints. No biochemical evidence is present on eventual impairment of kidney or liver function in humans. In contrast, in Han:SPRD-cy rats, an animal model of human polycystic kidney disease, creatinine clearance diminished and renal impairment was found to be accelerated upon creatine supplementation [12]. Exact determination of glomerular filtration rate (GFR) in small laboratory animals can be a cumbersome task [13]. Isotope-based methods and inulin clearance are still regarded as gold-standard methods. Creatinine clearance is easier to perform; however, creatinine is partly secreted by the tubules and analytical interferences exist in serum creatinine determination. The Jaffé reaction is influenced by protein, bilirubin, glucose and other non-specific chromogens [14]. The magnitude of the interference varies from method to method. Moreover, creatinine production is dependent on body composition and increases upon creatine supplementation. Several low-molecular-weight proteins have been described as good alternatives to serum creatinine in determining kidney function, independent of body composition. Cystatin C is a 13.3 kDa cysteine-protease inhibitor present in plasma. It has been regarded as the product of a ‘housekeeping’ gene and therefore production is considered to be stable. Cystatin C is freely filtered and neither reabsorbed intact nor secreted. Using anti-human cystatin C antibodies, cystatin C concentrations have been measured in rat sera with good linearity [15]. This study investigates the effects of creatine supplementation on kidney function in rats with normal kidney function and in rats with pre-existing renal impairment. Glomerular filtration was investigated using inulin and creatinine clearances, together with serum cystatin C concentration, 24 h protein excretion, urea and albumin clearance.

Subjects and methods

High-pressure liquid chromatography of the creatine formulation

Creatine monohydrate (99% pure) was obtained from Sigma (St Louis, MO, USA). Possible creatinine contamination of this formulation was assessed by high-pressure liquid chromatography (HPLC) analysis as described previously [16].

Experimental model of moderate chronic renal failure

Male Wistar rats weighing 200–230 g were obtained from Iffa Credo (Brussels, Belgium). The animals had free access to drinking water and were fed ad libitum. After acclimatization, animals were randomly allocated to four experimental groups: (i) sham-operated, normal diet (n=10); (ii) sham-operated, creatine fed (n=10); (iii) renal failure, control diet (n=12); and (iv) renal failure, creatine fed (n=11). All animal care was in accordance with local prescriptions and the NIH Guide for the Care and Use of Laboratory Animals.

Moderate renal failure was induced using a standard procedure of tissue removal (two-thirds nephrectomy), as described previously [17]. In brief, rats were anaesthetized with halothane (Fluothane; AstraZeneca, Södertälje, Sweden) and a flank incision was made exposing the kidney. The upper and lower pole of the left kidney were cryoablated, followed 1 week later by a right nephrectomy. Sham procedure consisted of flank incision and manipulation of the kidney without destruction of tissue. The cumulative mortality, including nephrectomy/sham procedure, was 14%, mostly due to post-operative bleeding or anaesthesia-associated deaths [17]. Renal failure was confirmed by a serum creatinine determination 1 week after the last incision. Dietary manipulation was started 1 week after last surgery/incision. Control animals received a soy-based grounded maintenance chow (RM1; Special Diet Services, Witham, UK) containing 14% protein. Creatine monohydrate (2% w/w) was added to this diet in the creatine-supplemented groups. Rats were housed in metabolic cages for 24 h during the study at two occasions: at the start and after 4 weeks of creatine supplementation. Urine and blood samples were collected and food intake noted on these occasions. Body weight was measured once a week. Creatine intake in the supplemented groups was calculated from food intake. Creatine intake in the control-diet groups is neglectible due to the absence of meat products in the diet.

Measurement of glomerular filtration rate and proteinuria

Creatinine clearance**.** Serum and urinary creatinine concentrations were determined using an enzymatic method (Roche Diagnostics, Mannheim, Germany) on a Hitachi 911 Autoanalyzer according to the

manufacturer's procedures. Creatinine clearances were calculated based upon 24 h urine collections.

Inulin clearance. After 4 weeks of creatine supplementation, animals (n=27) were anaesthetized using pentobarbital (5 mg/100 g) (Nembutal; Sanofi, Libourne, France) and placed on a temperature-controlled heating pad. The trachea was intubated, the left carotid artery was canulated using a PE50 catheter for repeated blood sampling and the left jugular vein was canulated for continuous saline administration at a rate matching diuresis. A single bolus (80 mg/kg) of fluorescein isothiocyanate (FITC)-inulin (Sigma) was administered intravenously and plasma samples were obtained at t=0, 3, 30, 120, 140, 160 and 180 min. The FITC-inulin concentration was determined using a 96-well fluorometer (Fluoroscan; Titertek, Huntsville, AL, 35805). Inulin clearance was calculated as described previously [13].

Cystatin C concentrations were measured using anti-human cystatin C antibodies (Dade Behring, Marburg, Germany) on a BN nephelometer (Dade Behring). Relative values are reported, expressed as mg/l of human cystatin C, as described previously [15]. Using this cystatin C assay, correlations between renal function indices were in accordance with published data (serum creatinine vs cystatin C: $r=0.92$, $P<0.001$; creatinine clearance vs 1/cystatin C: $r=0.94$, $P<0.001$; inulin clearance vs 1/cystatin C: $r=0.82$, $P<0.001$). Using these animal samples, the coefficient of variance (CV) value for this assay was 5.0%.

Serum and urine urea were determined enzymatically using Roche diagnostics kits on a modular autoanalyser. Urea clearances were calculated using 24 h urine collections. Serum albumin was determined using the bromocresol green method. Total urinary protein was measured by pyrogallol red. Urine albumin concentration was determined as described before using a Protur Hisi kit [18].

Tissue collection and processing

After the inulin clearance determinations, the animals were killed by cervical dislocation under pentobarbital anaesthesia and hindlimb muscles (soleus and gastrocnemius) were quickly excised, trimmed of fat and connective tissue, washed in phosphate-buffered saline (pH 7.4, 0.075 M), blotted dry, weighed and stored at -70°C for further analysis. Serum creatine was determined enzymatically [19] and total muscle creatine concentrations were determined colorimetrically [20].

Statistical analysis

Multiple-sample comparison was performed using Kruskal– Wallis testing. One-sample Kolmogorov–Smirnov test was used to pre-test normal distribution. Student's t-test (means±SD) or Mann–Whitney U-test [median (interquartile range)] were used to compare separate groups

when appropriate. Differences were considered significant at $P<0.05$. Correlation between parameters was examined using regression analysis.

Results

High-pressure liquid chromatography of the creatine formulation

The creatine monohydrate formulation (Sigma) used in this study for supplementation was found to contain 0.37% creatinine. No other side products were detected in the formulation.

Effect of creatine supplementation on glomerular filtration rate

No detrimental effect of creatine supplementation on kidney function could be observed during the study period. Table 1 summarizes serum and urine renal function indices. Serum creatinine concentrations were higher in two-thirds nephrectomized creatine-supplemented animals, compared with two-thirds nephrectomized control diet fed animals (0.72±0.19 vs. 0.58±0.08 mg/dl; $P<0.001$). Sham-operated animals did not differ in serum creatinine concentrations upon creatine supplementation. No significant difference was observed in serum urea or cystatin C concentrations or in 24 h protein excretion between supplemented and control-diet fed animals. Two-thirds nephrectomized animals had significantly different serum creatinine, cystatin C (Figure 1) and serum urea concentrations ($P<0.01$) and exhibited higher 24 h proteinuria [19.3±7.5 vs 15.1±4.7 mg; $P<0.05$ (pooled data)] and 24 h albuminuria [5.5±4.5 vs 1.9±1.2 mg; $P<0.01$ (pooled data)].

Table 1. Serum and urine renal function indices after 4 weeks of creatine supplementation

	Sham-operated		Two-thirds nephrectomized	
	Control diet ($n=10$)	Creatine loaded ($n=10$)	Control diet ($n=12$)	Creatine loaded ($n=11$)
Serum creatine (mg/dl)	1.78±0.91	9.21±4.73[a]	1.72±1.20	13.50±5.45[a]
Serum creatinine (mg/dl)[b]	0.43±0.12	0.39±0.15	0.58±0.08	0.72±0.19[a]
Serum urea (g/l)[b]	0.30±0.06	0.32±0.03	0.58±0.12	0.58±0.09
24 h urinary protein excretion (mg/24 h)[b]	16.3±4.4	13.8±4.8	19.3±8.2	19.3±7.3
24 h urinary albumin excretion (mg/24 h)[b]	2.4±1.4	1.4±0.7	5.0±4.2	5.9±4.9

Kruskal–Wallis multiple group comparison was significant for all parameters ($P \leqslant 0.001$).
[a] $P<0.05$, creatine-loaded animals compared with their respective control-diet fed animals.
[b] $P<0.05$, two-thirds nephrectomized animals had significantly different renal function indices compared with their respective controls. Serum creatine did not differ significantly between two-thirds nephrectomized and sham-operated animals.

Creatine-supplemented groups exhibited higher serum creatine concentrations [11.66±5.48 vs. 1.74±1.04 mg/dl; P<0.0001 (pooled data)]. No significant differences in creatine concentrations were observed between two-thirds nephrectomized and shamoperated animals for either creatine-supplemented or control-diet groups. Inulin and creatinine clearance rates, corrected for body weight and serum cystatin C concentrations, are illustrated in Figure 1. No differences were observed in either inulin, creatinine clearance [absolute values (data not shown) or relative to body weight] or serum cystatin C values (P=NS) between creatine-supplemented and control-diet fed animals. Glomerular filtration rate in two-thirds nephrectomized animals was significantly lower compared with sham-operated animals [inulin clearance: 0.21±0.04 vs 0.33±0.07 ml/min/100 g, P<0.001; creatinine clearance: 0.30±0.07 vs. 0.48±0.13 ml/min/100 g, P<0.001; cystatin C: 97±21 vs. 50±15 mg/l, P<0.001 (pooled data)]. Creatinine clearance rates at the start and after 4 weeks of creatine supplementation are illustrated in Table 2. Creatinine clearance was comparable at the start and after 4 weeks of creatine supplementation between supplemented and control-diet fed rats in either sham-operated or two-thirds nephrectomized animals. Recuperation of kidney function after renal ablation/nephrectomy was comparable in the creatinesupplemented group vs control-diet fed rats (0.69±0.34 vs. 0.61±0.30 ml/min; P=NS) in the renal failure group.

Table 2. Creatinine clearance during study period

	Days of creatine supplementation		
	$t=0$ days	$t=28$ days	P-value
Sham-operated			
Control diet ($n=10$)	2.45 ± 0.39	1.96 ± 0.60	NS
Creatine loaded ($n=10$)	2.59 ± 0.26	2.13 ± 0.58	
Two-thirds nephrectomized			
Control diet ($n=12$)	0.54 ± 0.19	1.15 ± 0.22	NS
Creatine loaded ($n=11$)	0.51 ± 0.31	1.17 ± 0.33	

Creatinine clearances are expressed as ml/min. Creatine-supplemented animals did not differ in creatinine clearance rate at the start or after 28 days of creatine supplementation ($P=$ NS).

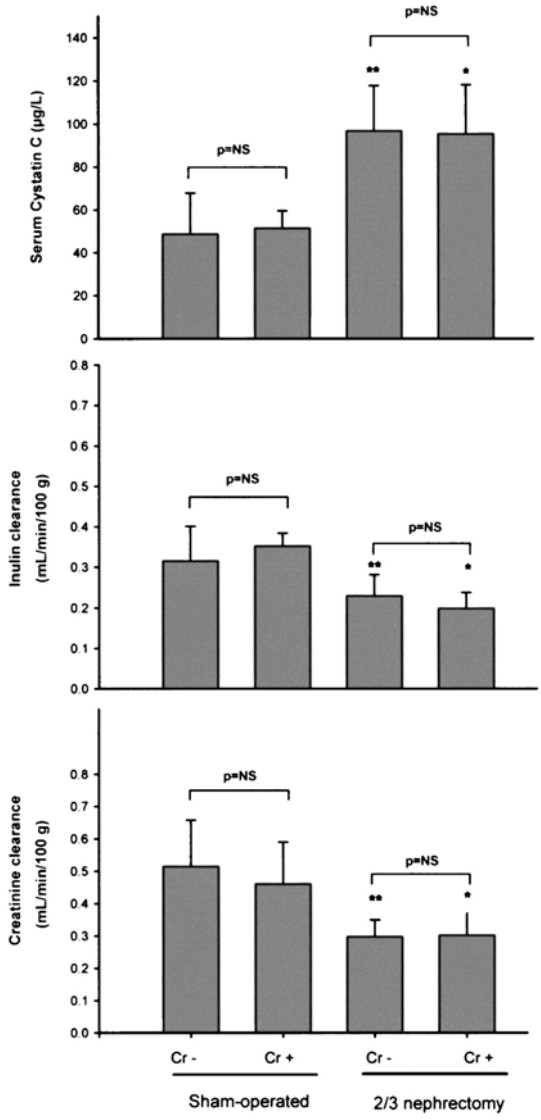

Fig. 1. Effect of 4 weeks of creatine supplementation on inulin, creatinine clearance and serum cystatin C concentrations. No significant differences in inulin, creatinine clearance and cystatin C concentrations were observed between supplemented animals and control-diet fed animals. Two-thirds nephrectomized animals had significantly different glomerular filtration indices compared with sham-operated animals. Cr-, control diet; Cr+, creatine-supplemented diet. *P<0.05, two-thirds nephrectomized supplemented animals, compared with supplemented sham-operated animals. **P<0.05, two-thirds nephrectomized control-diet fed animals, compared with control-diet fed sham-operated animals.

Effect of creatine supplementation on albumin and urea excretion

Urea and albumin clearance rates are illustrated in Figure 2. No differences were observed in either urea or albumin clearance between creatine-supplemented and control-diet fed animals [urea clearance: 0.21±0.06 vs. 0.18±0.07 ml/min/100 g, P=NS; albumin clearance: 0.019 (0.013–0.036) vs. 0.018 (0.011–0.042) ml/min/100 g, P=NS (Mann–Whitney U-test) (pooled data)].

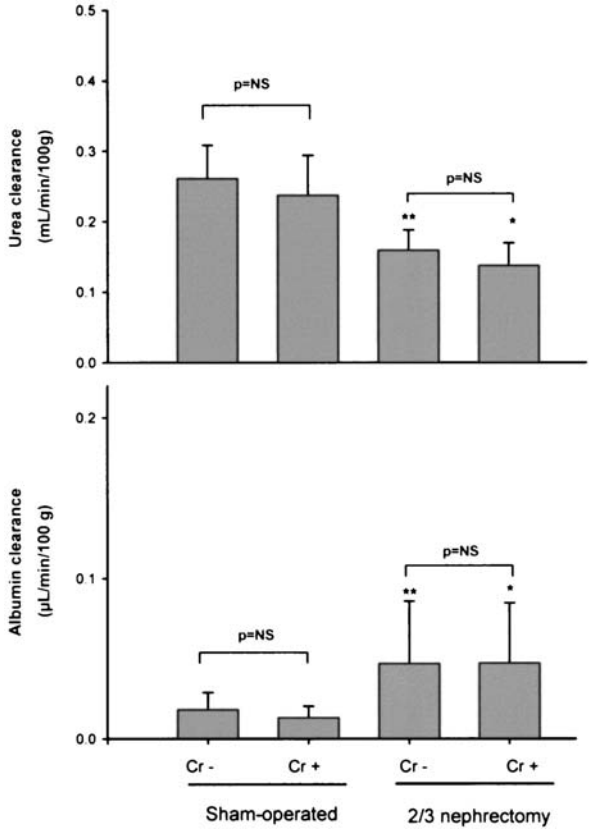

Fig. 2. Effect of 4 weeks of creatine supplementation on urea and albumin clearance rates. No significant differences in urea or albumin were observed between supplemented animals and control-diet fed animals. Two-thirds nephrectomized animals had significantly different urea and albumin clearance rates compared with sham-operated animals. Cr-, control diet; Cr+, creatine-supplemented diet. *P<0.05, two-thirds nephrectomized supplemented animals, compared with supplemented sham-operated animals. **P<0.05, two-thirds nephrectomized control-diet fed animals, compared with control-diet fed sham-operated animals.

Effect of creatine supplementation on body mass, food intake and muscular creatine concentrations Table 3 summarizes food intake and body mass in the four experimental groups. No difference in body weight or food intake was observed after 4 weeks of creatine supplementation in either sham-operated or in the two-thirds nephrectomized group. Two-thirds nephrectomized animals exhibited lower body mass than sham-operated controls [387±22 vs. 421±21 g; P<0.001 (pooled data)]. Daily food was not different between sham-operated and two-thirds nephrectomized animals [17.5±2.6 vs. 17.4± 3.7 g; P=NS (pooled data)]. Creatine intake, expressed as absolute intake (353±69 vs. 333±85 mg; P=NS) or relative to body weight (853±173 vs. 870±242 mg/kg; P=NS) in supplemented animals did not differ between two-thirds nephrectomized and sham-operated animals.

Total creatine intramuscular concentrations are increased upon supplementation [soleus: 23.9±4.0 vs. 21.5±2.3 μmol/g, P=0.02; gastrocnemius: 35.8±2.9 vs 32.8±1.6 μmol/g, P<0.001 (pooled data)]. No difference in muscular creatine concentration was observed comparing two-thirds nephrectomized animals with sham-operated animals [soleus: 24.0±4.2 vs 21.9±2.6 μmol/g, P=NS; gastrocnemius: 34.4±2.6 vs 34.5±3.3 μmol/g, P=NS (pooled data)].

Table 3. Effect of 4 weeks of creatine supplementation on body mass, food intake and total creatine intramuscular concentration

	Sham-operated		Two-thirds nephrectomized	
	Control diet ($n=10$)	Creatine loaded ($n=10$)	Control diet ($n=12$)	Creatine loaded ($n=11$)
Body mass (g) ($t=0$ days)	259±15	262±16	250±11	252±13
Body mass (g) ($t=28$ days)[a]	416±23	426±18	389±22[b]	386±23[b]
Food intake (g) ($t=0$ days)	17.3±2.5	18.5±2.0	17.2±3.2	17.8±2.4
Food intake (g) ($t=28$ days)	17.2±1.3	17.7±3.4	18.2±2.9	16.6±1.2
Total creatine, soleus (μmol/g)	22.1±1.9	25.4±5.0	21.0±2.5	22.7±2.5
Total creatine, white gastrocnemius (μmol/g)[a]	32.2±0.9	35.9±3.5[c]	33.1±1.9	35.8±2.3[c]

Creatine concentrations are expressed as μmol/g wet weight.
[a] $P<0.05$, Kruskal–Wallis multiple group comparison.
[b] $P<0.01$, two-thirds nephrectomized animals compared with their respective sham-operated control animals.
[c] $P<0.01$, creatine loaded animals compared with their respective control-diet fed animals.

Discussion

The present study could not demonstrate any deleterious effect of creatine supplementation on kidney function. No changes in GFR or renal protein handling were observed. Creatine supplementation was shown to increase intramuscular total creatine concentrations in sham-operated and nephrectomized animals.

Creatinine determinations are susceptible to analytical interferences. These in vitro interferences can make up a substantial fraction of the apparent creatinine concentrations. The Jaffe´ reaction is particularly susceptible to non-specific chromogens, such as bilirubin and cephalosporins [14]. Creatine supplementation can interfere with creatinine determinations by Jaffé method, with apparent increases in creatinine concentrations up to 8% (Y. Taes, unpublished data). No analytical interferences were found using the enzymatic assay with increased creatine concentrations, as found in animals or subjects who ingest creatine (Y. Taes, unpublished data). As creatine feeding could interfere with creatinine determination, we investigated GFR by means of the gold-standard procedure (inulin clearance), together with creatinine clearance and serum cystatin C. No significant alterations in any of the markers were observed upon creatine supplementation, indicating no effect on GFR. Urea clearance was comparable in creatine-supplemented and control-diet animals. No changes in 24 h proteinuria or albumin excretion were noted in both sham-operated and two-thirds nephrectomized animals upon creatine supplementation, indicating absence of structural glomerular changes. Serum creatinine concentrations were found to be higher in nephrectomized supplemented animals but not in creatine-supplemented sham-operated animals. With declining kidney function, increased serum creatinine concentrations are attained in order to excrete the increased creatinine load, due to creatine– creatinine interconversion. However, care is warranted as creatinine has been described as a weak uraemic toxin. Inhibitory effects on metabolic processes have been described, however, at supraphysiological concentrations and not observed in vivo [21].Upon creatine supplementation, Edmunds et al. [12] demonstrated renal disease progression in Han:SPRD-cy rats. These authors described increases in serum creatinine concentrations in male (but not in female) rats. Unfortunately, no independent renal function measurements were performed. Creatinine clearances were diminished in both sexes. Creatinine was determined using an end point Jaffé-based method (alkaline picrate; Sigma Diagnostics). The Jaffé method is susceptible to interference from non-specific chromogens [14], varying from method to method. The creatinine values reported by Edmunds et al. [12] might have been influenced by creatine concentrations. Unfortunately, no creatine determinations were communicated in the former study. Creatine was supplemented as a mixture of creatine and glutamine in an over-the-

counter formulation. This formulation could contain traces of contaminants or toxic products. Purity of this formulation has not been established.

The Han:SPRD-cy rat is a well-established animal model of human polycystic kidney disease. Several interventional studies reported effects on kidney function in this animal. Dietary interventions have been shown to delay the progression of renal disease [22]. While this model mimics human polycystic disease well, it cannot be used as an animal model for general renal functional impairment. The remnant kidney model is a well-established animal model for kidney function impairment, by means of kidney tissue ablation. Intrinsic renal disease is, however, absent in these animals and the kidney function can partly recover over time. These differences in animal model can account for the differences found between the present study and the study by Edmunds et al. [12]. In humans, no evidence from controlled studies is available describing adverse effects of creatine supplementation. Poortmans [5,8,9,11] investigated kidney function in young healthy subjects. No alterations in creatinine, urea or albumin clearance were reported in short-term (5 days, 20 g creatine daily [8]) or long-term studies (1 month to 5 years, with 1–80 g creatine daily [9]; 63 days with 21 g creatine daily [11]). Robinson et al. [10] reported only transient increases in serum creatinine during creatine ingestion. Six weeks after cessation of the creatine supplementation, serum creatinine had returned to baseline. No gold-standard procedures for determining GFR, such as inulin or isotope-based methods, have been performed in humans. Creatine is regarded as a nutritional supplement and available over-the-counter. The different industrially prepared formulations are not subjected to adequate quality control. Toxic side products or contaminants could be present in these formulations. No toxic side products were detected in the creatine monohydrate formulation by our HPLC method [16]. Creatinine contamination was found to be 0.37%. Intake of creatinine is therefore very low in comparison to creatine intake. This study demonstrates that creatine supplementation can increase intracellular creatine concentration in skeletal muscle of renal failure animals, as described in animals with normal kidney function [1]. Serum and muscle creatine concentrations were found to increase upon creatine supplementation in animals with kidney function impairment. Creatine supplementation in animals has been shown to increase intracellular creatine concentration (total, free and phosphocreatine) in both fast-twitch and slow-twitch skeletal muscles, together with an improved running performance [1]. Differences in creatine concentrations between the soleus and gastrocnemius are in accordance with reported values [1], reflecting differences in energy metabolism. No intramuscular phosphocreatine or high energy nucleotides are reported in the present study as the prolonged anaesthesia during inulin clearance determination could have influenced these high energy metabolites.

In conclusion, we could not demonstrate any harmful effects of prolonged high dose creatine supplementation on glomerular filtration or protein excretion in an animal model with pre-existing moderate renal dysfunction. Muscle intracellular creatine concentration was increased upon creatine supplementation in these animals.

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Note added to manuscript in thesis:

The provided analgesia in relation to surgery in the animals is not mentioned in the manuscript. The analgesia was provided 48h post-operatively, by administration of buprenorphine IM (0.1 mg/kg; Temgesic, Schering-Plough)

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CHAPTER 3.2

Effect of creatine supplementation on homocysteine metabolism in animals and chronic hemodialysis patients

Creatine supplementation decreases homocysteine in an animal model of uraemia.
Kidney Int 2003;64:1331-1337

Creatine supplementation does not decrease total plasma homocysteine in chronic hemodialysis patients.
Kidney Int 2004;66:2422-2428

Creatine supplementation decreases homocysteine in an animal model of uraemia

Taes YEC, et al.
Kidney Int 2003;64:1331-1337

Abstract

Background. Hyperhomocysteinemia is prevalent in more than 85% of patients with end-stage renal disease (ESRD) and is thought to contribute to the excess cardiovascular mortality and morbidity. Creatine is synthesized by methylation of guanidinoacetate with formation of S-adenosylhomocysteine and subsequently, homocysteine (Hcy). Creatine supplementation down-regulates its endogenous synthesis and, thus, may reduce Hcy production. The present study investigates the effect of creatine supplementation on Hcy concentrations in an animal model of uraemia.

Methods. Male Wistar rats were either sham-operated and received a control diet (N=8) or a 2%-creatine-supplemented diet (N=8), or underwent subtotal nephrectomy and received a control diet (N=10) or a 2%-supplemented creatine diet (N=10). After 2 weeks of treatment, total plasma Hcy, creatine, creatinine, folate, and vitamin B12 were determined, as well as hepatic folate and vitamin B12 concentrations.

Results. Plasma creatinine concentrations were higher in nephrectomized animals, but similar in creatine-supplemented and control diet–fed animals. Plasma Hcy was higher in nephrectomized animals but lower in creatine-supplemented nephrectomized animals compared to nephrectomized control diet–fed animals (12.1 ± 2.4 μmol/L vs. 15.4 ± 1.7 μmol/L; $P < 0.01$). Total plasma Hcy inversely correlated with plasma creatine concentrations ($r = 0.39$; $P = 0.02$). Plasma folate was higher in supplemented animals and hepatic tetrahydrofolate (THF) was higher in nephrectomized supplemented animals. Plasma vitamin B12 was similar in all groups, whereas hepatic vitamin B12 was higher in nephrectomized animals.

Conclusion. Creatine supplementation can effectively lower plasma Hcy concentrations in an animal model of uraemia and should be further investigated as a potential treatment for hyperhomocysteinemia in patients with ESRD.

Key words: total plasma homocysteine, tetrahydrofolate, 5-methyltetrahydrofolate, end-stage renal disease, creatine.

Introduction

Hyperhomocysteinemia is considered an independent risk factor for cardiovascular disease in the general population, as well as in patients with end-stage renal disease (ESRD) [1, 2]. About 85% of patients with renal failure have hyperhomocysteinemia, eventually contributing to the excess cardiovascular mortality in this population [3]. The mechanism underlying hyperhomocysteinemia in ESRD is poorly understood. Homocysteine (Hcy) [2] is a sulfur-containing amino acid, formed by demethylation of methionine and plays an important role in the activated methyl and folate cycle (Fig. 1).

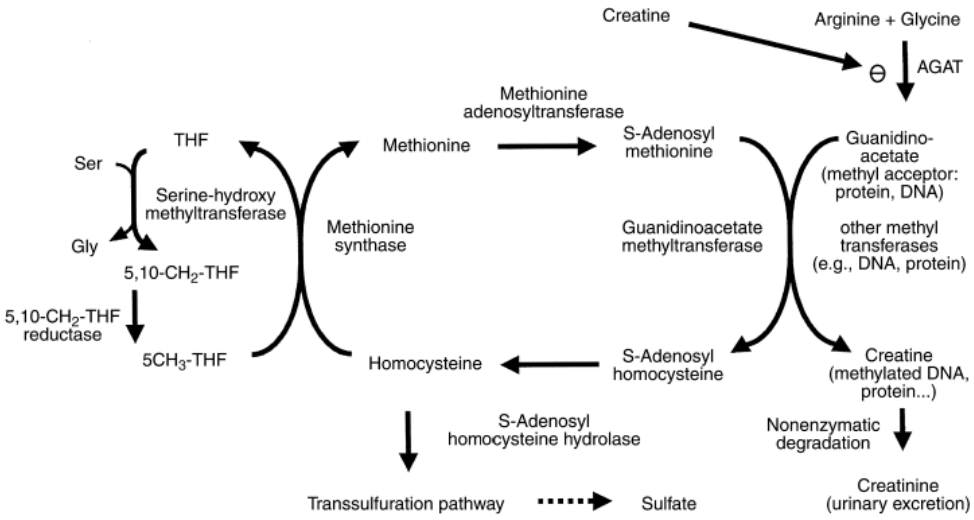

Fig. 1. Overview of homocysteine and creatine metabolism. Methionine is converted to S-adenosylmethionine, which is demethylated to S-adenosylhomocysteine by methyltransferases. Creatine is formed by methylation of guanidinoacetate, which is synthesized from glycine and arginine. Creatine supplementation decreases guanidinoacetate synthesis by repressing AGAT biosynthesis and consequent reduction of guanidinoacetate formation. Homocysteine is either remethylated to methionine or degraded in the transsulfuration pathway. Abbreviations are: AGAT, arginine:glycine amidinotransferase (EC 2.1.4.1); THF, tetrahydrofolate; $5CH_3$-THF, 5-methyltetrahydrofolate; 5,10-CH_2-THF: 5,10 methylene tetrahydrofolate; Ser, serine; Gly, glycine.

Methionine can intracellularly be converted to S-adenosylmethionine, a sulfonium compound with a highly reactive methyl group, which acts as a universal methyl donor in numerous transmethylation reactions in vivo. The demethylated product S-adenosyl homocysteine, a thioether, is readily hydrolyzed to Hcy and adenosine by S-adenosyl homocysteine hydrolase. Hcy metabolism is nutritionally regulated. In conditions with high methionine concentrations, the transsulfuration pathway is favored, in which Hcy condenses with serine to cystathionine, which is further metabolized to cysteine and sulfate. In conditions of low methionine intake, remethylation of Hcy to methionine occurs through methyl donation from 5-methyltetrahydrofolate (5-CH_3THF) by means of methionine synthase. Vitamin B12 acts as a cofactor in this remethylation pathway. 5-CH_3THF is demethylated to tetrahydrofolate (THF), which is reconverted to 5-CH_3THF in a multiple-step folate cycle with serine glycine conversion by means of the methylene tetrahydrofolate reductase. Treatment of hyperhomocysteinemia mainly consists of alimentary folic acid and vitamin B6 and B12 supplements. An average reduction of about 30% in plasma total Hcy (tHcy) is observed in uraemic patients treated with folic acid [3]. The effect of vitamin B6 and B12 is in general much less pronounced [3]. A randomized analysis by Bostom et al [4] reported a decrease of 12% in fasting plasma tHcy with vitamin B6 supplementation. Vitamin B12 supplementation lowered tHcy concentration with 35%, only in dialysis patients with low cobalamin status [5]. However, even combined high-dose folic acid and vitamin B6 or B12 supplementation cannot entirely normalize tHcy in ESRD patients [3]. Supplementation with other methyl-donating molecules such as betaine can decrease postmethionine loading tHcy concentrations by 18%, in addition to folic acid supplementation [6]. Additional tHcy lowering therapies are required in preventing excess cardiovascular mortality in these patients.

Creatine is increasingly popular as an ergogenic supplement in an athletic population [7 9]. Dietary intake and endogenous synthesis in liver compensate for a daily loss as creatinine of about 2%. Creatine is synthesized in mammals through two successive metabolic steps (Fig. 1). First, guanidinoacetate is formed in the kidney from glycine and arginine by arginine:glycine amidinotransferase (AGAT) (EC 2.1.4.1). Second, guanidinoacetate is methylated in the mammalian liver to creatine by guanidinoacetate methyltransferase (GAMT) (EC 2.1.1.2) with S-adenosylmethionine acting as methyl donor [8]. Creatine supplementation down-regulates endogenous creatine synthesis by repression of AGAT biosynthesis and consequent guanidinoacetate formation (Fig. 1).

Mudd and Poole [10] and Mudd, Ebert, and Scriver [11] calculated that methylation of guanidinoacetate during creatine biosynthesis accounted for up to 70% of the transmethylation reactions in the body. Quantitatively, this exceeds all other methylation reactions. Stead et al [12] demonstrated

that down-regulation of endogenous creatine synthesis by creatine supplementation could decrease methylation demand and tHcy in animals with normal renal function. Whether creatine can also lower tHcy in conditions of renal failure is presently unknown.

The present study investigates the effect of creatine supplementation on tHcy concentrations in both animals with uraemia and normal kidney function.

Methods

Animal procedures

Male Wistar rats weighing 200 to 230 g were obtained from Iffa Credo (Brussels, Belgium). All animal care was in accordance with local prescriptions and the NIH Guide for the Care and Use of Laboratory Animals.

Renal failure was induced using a standard procedure of subtotal nephrectomy as described before [13, 14]. Rats were anesthetized with halothane (Fluothane) (Astra-Zeneca, Destelbergen, Belgium) and a flank incision was made exposing the kidney. The upper and lower poles of the left kidney were cryoablated, followed 1 week later by a right nephrectomy. The sham procedure consisted of a flank incision and manipulation of the kidney without tissue destruction.

Animals were randomly allocated to four groups: (1) sham-operated, control diet (N = 8); (2) sham-operated, creatine-supplemented diet (N = 8); (3) subtotal nephrectomized, control diet (N = 10); and (4) subtotal nephrectomized, creatine-supplemented diet (N = 10). The animals had free access to food and drinking water.

Creatine supplementation was started 1 week after the last surgical procedure by addition of 2% creatine monohydrate (wt/wt) (Sigma Chemical Co., St. Louis, MO, USA) to the control diet. Control animals were kept on a soy-based maintenance diet (RM1) (Special Diet Services, Witham, UK), containing 14% protein.

Urine and blood samples were collected at the start and after 14 days of creatine supplementation. Body weight and individual food intake were recorded on these occasions. Folate intake was calculated from food intake.

Biochemical determinations

Creatinine clearances. Plasma and urinary creatinine concentrations were determined enzymatically (Roche Diagnostics, Mannheim, Germany) on a Modular P analyzer (Roche Diagnostics) according to the manufacturer's procedure. Creatinine clearances were calculated based upon 24-hour urine collections. Plasma urea concentrations were determined enzymatically (Roche Diagnostics).

Total plasma Hcy concentrations were determined using a fluorescence polarization immunoassay on an Axsym analyzer (Abbott Laboratories, Abbott Park, IL, USA).

Plasma folate and plasma and hepatic vitamin B12 concentrations were determined using an electrochemiluminescence assay (Roche Diagnostics) on an Elecsys 2010 Analyzer (Roche Diagnostics). Plasma and urine creatine concentrations were determined enzymatically as described before [15]. Total intramuscular creatine concentrations (mol/wet weight) were determined colorimetrically according to Berlet [16]. Urinary sulfate was determined colorimetrically using barium chloranilate according to Bertolacini and Barney [17]. Hepatic nonsubstituted THF and 5-CH_3THF (monoglutamates) were determined using high-pressure liquid chromatography (HPLC) with fluorometric detection as described before [18]. Samples were homogenized in 50 mmol/L acetate buffer (pH 4.9) (2% ascorbic acid and 1% 2-mercaptoethanol), deconjugated using rat plasma and heated for 5 minutes in boiling water. Samples were flushed with nitrogen between every manipulation. The analysis was performed on Gilson 307 HPLC pump (Gilson Int., Den Haag, The Netherlands) with a Gilson 122 fluorometric detector (ex, 295 nm; em, 356 nm) using a Chrompack Spherisorb 3 ODS-2 reversed-phase column. Recoveries were 86% and 93% for THF and 5-CH_3THF, respectively.

Statistics

Data are expressed as mean ± SD. One-sample Kolmogorov-Smirnov test was used to pretest normal distribution. Student t test was used to compare separate groups when appropriate. Differences were considered significant at P less than 0.05. Correlation between parameters was examined using Spearman rank correlation analysis.

Results

Biometrical and biochemical characteristics

Subtotal nephrectomized animals exhibited lower body mass and renal function indices compared to sham-operated animals. No significant differences in body weight, food, or folate intake were observed between creatine-supplemented and control diet fed groups in either subtotal nephrectomized or sham-operated groups (Table 1). Creatinine clearance rates were similar in creatine-supplemented and control diet fed animals (Table 1). Plasma creatine and urinary creatine excretion was significantly higher in creatine-supplemented animals. Total intramuscular creatine concentrations were similar in nephrectomized and sham-operated animals [34.5 ± 2.6 µmol/g vs. 34.8 ± 3.3 µmol/g; P = NS (pooled data)], but higher concentrations were observed in creatine-supplemented animals than in control diet fed animals [36.1 ± 2.8 µmol/g vs. 33.0 ± 2.0

µmol/g; P = 0.001 (pooled data)]. Urinary sulfate excretion did not differ between creatine-supplemented and control diet fed animals.

Table 1. Comparative biometric characteristics, baseline glomerular filtration markers, and biochemical parameters in control diet (soy-based) and creatine-supplemented (2% wt/wt) diet–fed animals, with or without preexisting renal failure

	Sham-operated		Subtotal nephrectomized	
	Control diet ($N = 8$)	Creatine diet ($N = 8$)	Control diet ($N = 10$)	Creatine diet ($N = 10$)
Body weight *g*	363 ± 21	372 ± 19	331 ± 17[a]	341 ± 20[a]
Food intake *g/day*	17 ± 1	18 ± 4	18 ± 3	17 ± 5
Folate intake *mg/day*	0.017 ± 0.001	0.018 ± 0.004	0.018 ± 0.003	0.017 ± 0.005
Plasma creatinine *µmol/L*	20.0 ± 3.4	20.0 ± 3.9	50.7 ± 7.8[a]	48.4 ± 9.0[a]
Plasma urea-N *mmol/L*	10.7 ± 2.5	11.8 ± 1.4	21.1 ± 4.3[a]	20.7 ± 3.2[a]
Creatinine clearance *mL/min/100 g*	0.70 ± 0.13	0.79 ± 0.19	0.18 ± 0.07[a]	0.16 ± 0.10[a]
Plasma creatine *µmol/L*	124 ± 62	760 ± 410[b]	134 ± 95	935 ± 371[b]
Urinary creatine *µmol/day*	0.86 ± 0.13	1537 ± 943[b]	0.63 ± 0.05	1508 ± 788[b]
Total muscular creatine *µmol/g*	32.9 ± 2.2	36.2 ± 3.4[b]	33.1 ± 2.0	36.0 ± 2.4[b]
Urinary sulfate *µmol/day/kg*	106.3 ± 13.3	98.2 ± 18.2	140.2 ± 41.9	114.1 ± 67.4

[a]$P < 0.005$, subtotal nephrectomized group vs. respective sham-operated control group
[b]$P < 0.05$, creatine diet group vs. respective control diet–group

Plasma tHcy concentrations

Subtotal nephrectomized, control diet fed animals had higher tHcy concentrations compared to sham-operated control diet fed animals (Fig. 2). Lower tHcy concentrations were observed in nephrectomized creatine-supplemented animals compared to nephrectomized control diet fed animals (Fig. 2). In sham-operated animals, only a tendency toward lower tHcy concentrations was observed. Pooled data from sham-operated and nephrectomized animals demonstrated significant lower tHcy concentrations in the creatine-supplemented groups compared to the control diet fed group (12.4 ± 2.2 µmol/L vs. 14.6 ± 1.9 µmol/L; P = 0.005).

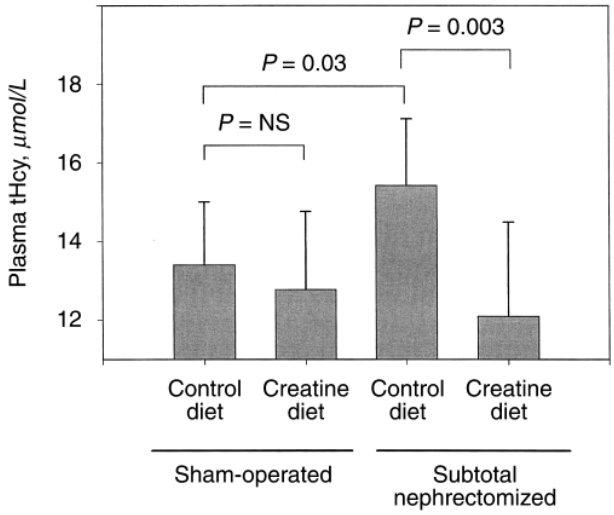

Fig. 2. Effect of creatine supplementation on total plasma homocysteine (tHcy) concentrations in sham-operated and subtotal nephrectomized animals. Subtotal nephrectomized animals had significantly higher tHcy concentrations than sham-operated animals. The creatinesupplemented subtotal nephrectomized group had lower tHcy concentrations, compared to nephrectomized control diet–fed group. Creatine supplementation in the sham-operated group did not lower tHcy concentrations significantly.

Plasma tHcy concentrations inversely correlated with plasma creatine concentrations ($r = 0.39$; $P = 0.02$) (Fig. 3), as well as with total intramuscular creatine concentrations ($r = 0.35$; $P < 0.05$).
tHcy concentrations correlated significantly with creatinine clearance in control diet fed animals ($r = 0.68$; $P = 0.005$), but the correlation was lost in creatine-supplemented animals ($r = 0.06$; P = NS) (Fig. 4).
No correlation between tHcy and urinary sulfate excretion was observed.

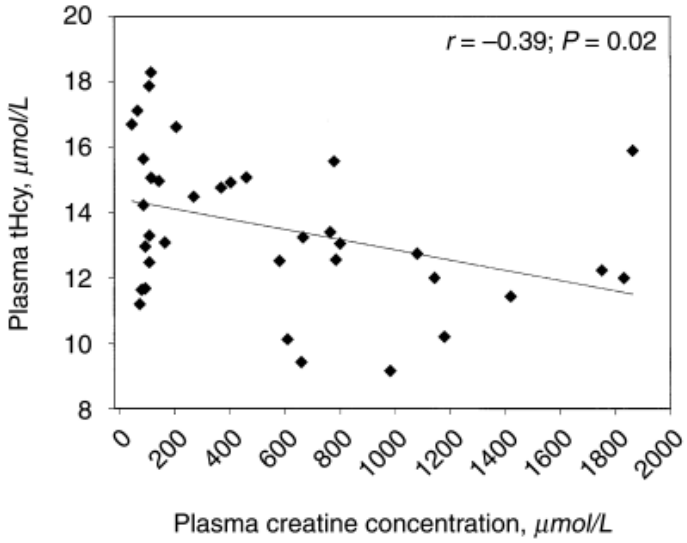

Fig. 3. Scatter diagram illustrating relation between plasma creatine and total plasma homocysteine (tHcy) concentrations. tHcy concentrations inversely correlated with plasma creatine concentrations. Data from all groups are included in the graph.

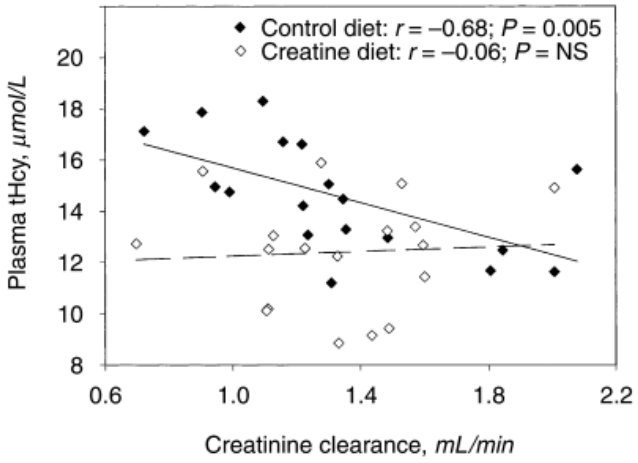

Fig. 4. Effect of creatine supplementation on the correlation between total plasma homocysteine (tHcy) concentrations and creatinine clearance (mL/min). tHcy concentrations correlated with creatinine clearance in control diet–fed animals, but the correlation was lost in creatinesupplemented animals. Data from all groups are included in the graph.

Plasma folate and vitamin B12 concentrations

No differences in plasma folate or vitamin B12 were observed between nephrectomized and sham-operated animals (Table 2). Plasma folate was higher in creatine-supplemented groups [70.0 ± 24.9 nmol/L vs. 40.8 ± 17.6 nmol/L; P = 0.001 (pooled data)]. No differences in plasma vitamin B12 concentrations were observed between creatine-supplemented and control diet fed animals [643 ± 121 pmol/L vs. 618 ± 165 pmol/L; P = NS (pooled data)]. No significant correlation was observed between plasma folate or vitamin B12 concentrations and plasma tHcy concentrations. Plasma creatine concentration correlated significantly with plasma folate (r = 0.47; P = 0.01) but not with vitamin B12 (r = 0.03; P = 0.87) concentrations.

Table 2. Effect of creatine supplementation on plasma, hepatic folate, and vitamin B12 concentrations, in animals with with or without preexisting renal failure

	Sham-operated		Subtotal nephrectomized	
	Control diet ($N = 8$)	Creatine diet ($N = 8$)	Control diet ($N = 10$)	Creatine diet ($N = 10$)
Plasma folate *nmol/L*	45.8 ± 17.2	72.6 ± 18.0[b]	38.0 ± 18.2	67.5 ± 31.3[b]
Plasma vitamin B_{12} *pmol/L*	521 ± 153	610 ± 62	672 ± 153	671 ± 155
Hepatic THF *nmol/g*	9.5 ± 2.7	10.6 ± 2.7	7.8 ± 3.1	11.4 ± 3.5[b]
Hepatic 5-CH_3THF *nmol/g*	12.2 ± 3.3	10.7 ± 2.1	10.3 ± 2.6	11.2 ± 2.7
Hepatic vitamin B_{12} *pmol/g*	48.5 ± 6.9	45.4 ± 5.4	72.0 ± 16.3[a]	67.9 ± 17.6[a]

Abbreviations are: THF, tetrahydrofolate; 5-CH_3THF, 5-methyltetrahydrofolate.
[a] $P < 0.05$, subtotal nephrectomized group vs. respective sham-operated control group
[b] $P < 0.05$, creatine diet group vs. respective control diet–group

Hepatic folate and vitamin B12 concentrations

Sham-operated and subtotal nephrectomized animals had similar hepatic THF and 5-CH_3THF concentrations. Hepatic vitamin B12 concentrations were higher in subtotal nephrectomized animals compared to sham-operated animals [7.0 ± 1.7 pmol/g vs. 4.7 ± 0.6 pmol/g; P = 0.002 (pooled data)]. Creatine-supplemented animals had higher hepatic THF concentrations [11.1 ± 3.2 nmol/g vs. 8.6 ± 2.9 nmol/g; P = 0.02 (pooled data)]. Hepatic 5-CH_3THF and vitamin B12 concentrations were comparable between creatine-supplemented and control diet fed animals.

Discussion

Uraemic animals have higher tHcy than animals with normal renal function, an observation in agreement with the well-known high prevalence of hyperhomocysteinemia in patients with ESRD [19]. The correlation of plasma tHcy with creatinine clearance in rats is in accordance to literature [20]. The salient observation of the present study is that oral creatine

supplementation can effectively lower plasma tHcy concentrations in animals with uraemia to normal tHcy concentrations. tHcy concentrations were 22% lower in the creatine-supplemented subtotal nephrectomized group, compared to the control diet fed group. Creatine supplementation in the sham-operated animals did not lower tHcy significantly. The latter observation is in line with the absence of an effect of creatine supplementation on tHcy in young healthy volunteers [21]. Hcy metabolism is tightly regulated under normal conditions, whereas in situations with compromised Hcy homeostasis, creatine supplementation might have a more direct and prominent tHcy lowering effect [22]. Species-related differences in metabolic pathways could also explain the difference in effect between humans and rodents.

Plasma, urine, and muscle creatine concentrations were higher in creatine-supplemented animals, documenting uptake and metabolization of creatine in this animal model. No difference in creatinine clearance was observed between supplemented and control diet fed animals in both sham-operated and nephrectomized animals. The safety of creatine supplementation and the absence of adverse effects on renal function were previously assessed by our group in this animal model of chronic renal failure [13]. No adverse effects on glomerular filtration or renal protein handling were observed. However, adverse effects of creatine supplementation on renal function have been reported. Edmunds et al [23] demonstrated diminished creatinine clearance rates and increased cyst formation in Han:SPRD-cy rats, an animal model of human polycystic kidney disease. So far, none of the controlled studies in humans on the effects of creatine supplementation have indicated any adverse effect [24]. However, safety of creatine supplementation in subjects with preexisting renal failure remains open for discussion.

Creatine supplementation was associated with higher plasma folate concentrations and higher hepatic THF concentrations in the subtotal neprectomized group, whereas folate intake was comparable in all groups. Higher folate concentrations in the creatine-supplemented nephrectomized animals reflect differences in folate metabolism, as folic acid intake is comparable. Creatine supplementation lowers endogenous creatine synthesis and methylation demand, with lower Hcy formation. Remethylation of Hcy to methionine might be diminished with an apparent folate-saving effect. However, further research is required confirming these hypotheses, eventually using stable isotope protocols. In contrast to humans [25], no relationship between urinary sulfate excretion and tHcy was observed in our animal model, probably due to similar dietary intake of methionine and cysteine in all animals, accounting for comparable sulfate excretion.

The influence of creatine on labile methyl pool metabolism, and consequently on methionine Hcy cycling and tHcy concentrations, has so far been underestimated. Estimation of labile methyl pool consumption

[10, 11] demonstrated that creatine biosynthesis accounts for up to 70% of the methylation reactions in the human body. The correlation between creatinine and tHcy concentrations is attributable to two factors. First, both tHcy and plasma creatinine depend on renal elimination. Moreover, creatine synthesis is intricately linked to Hcy formation and creatine is degraded to creatinine. The correlation observed between tHcy and creatinine is partly due to glomerular filtration, but also due to creatine creatinine conversion.

In humans, a low dietary intake of creatine is associated with higher tHcy concentrations. Vegetarians, consuming neglectable amounts of creatine, have higher tHcy than subjects consuming an omnivorous diet [26-28], even when vitamin B12 concentrations are similar [26]. Renal patients are generally kept on a low protein diet with low dietary creatine intake. Endogenous creatine synthesis and methylation demand could, therefore, be higher in these patients, enhancing tHcy concentrations.

In middle-aged and elderly subjects, tHcy was found to depend on gender with lower tHcy concentrations in women than in men [29]. Gender transition studies in male or female transsexuals have shown a strong dependency of tHcy on creatinine concentrations. Male-to-female transsexuals exhibited a substantial reduction in tHcy and creatinine concentrations after estrogen and antiandrogen therapy. Female-to-male transsexuals exhibited higher tHcy and creatinine concentrations after testosterone treatment. Taken together, these studies suggest that Hcy production occurs in direct conjunction with creatine synthesis [30].

Exogenous methylation demand, defined as increased metabolic methyl group consumption by intake of substances (alimentary, drugs) requiring methylation during their metabolization, can increase tHcy concentrations by increasing methionine Hcy interconversion. The effect of exogenous methylation demand on tHcy is illustrated in Parkinson disease patients, treated with L-3,4-dihydroxyphenylalanine (L-DOPA). Metabolism of L-DOPA involves the enzyme catechol-O-methyltransferase, with S-adenosylmethionine as methyl group donor with formation of S-adenosylhomocysteine. In Parkinson disease patients treated with L-DOPA, plasma tHcy is higher than in controls and untreated patients [31]. Increased exogenous methylation demand is also observed with the lipid-lowering agent niacin, which is metabolized and excreted as methylated pyridones. High-dose niacin intake imposes a large methylation demand on the activated methyl cycle and increases tHcy concentrations and decreases folate and vitamin B12 concentrations [32, 33].

Conclusion

The present study demonstrated that inhibition of endogenous methylation demand with dietary creatine supplementation can effectively lower tHcy in an animal model of uraemia. Creatine may thus have a promising

therapeutic potential in lowering tHcy concentrations and reducing cardiovascular risk in ESRD patients. Further research is required to examine whether the present results can be extrapolated to humans with ESRD.

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Note added to manuscript in thesis:

The provided analgesia in relation to surgery in the animals is not mentioned in the manuscript. The analgesia was provided 48h post-operatively, by administration of buprenorphine IM (0.1 mg/kg; Temgesic, Schering-Plough).

Blood samples were drawn after 3h fasting to minimize the influence of creatine uptake from the gut on plasma creatine concentrations, though the pharmacokinetic behaviour of creatine in this animal model was not studied.

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Creatine supplementation does not decrease total plasma homocysteine in chronic hemodialysis patients

Taes YEC, et al.
Kidney Int 2004;66:2422-2428

Abstract

Background. Hyperhomocysteinemia is present in the majority of chronic hemodialysis patients. Treatment with folic acid, vitamin B12, and vitamin B6 cannot fully normalize plasma homocysteine concentrations (tHcy). Previously we have demonstrated the tHcy-lowering effect of creatine supplementation in an animal model of uraemia (Kidney Int 64:1331-1337, 2003). The present study investigates the effects of creatine supplementation on tHcy in a vitamin-repleted chronic hemodialysis population.

Methods. Forty-five hemodialysis patients receiving folic acid and vitamin B6 and B12 were included. Patients were treated with creatine (2 g/day) or placebo during 2 treatment periods of 4 weeks, separated by a washout of 4 weeks. Plasma tHcy, creatine, Kt/V_{urea}, folic acid, vitamin B12, and routine biochemistry were determined, as well as the prognostic inflammatory and nutritional index.

Results. All patients had elevated tHcy concentrations (21.2 ± 5.6 mol/L). Creatine treatment resulted in increased plasma and red blood cell creatine levels, documenting uptake of creatine. Creatine did not affect tHcy concentrations. There was no relationship between plasma creatine concentrations and tHcy concentrations. No changes in body weight, routine biochemistry, nutritional status, folic acid, or vitamin B12 were observed during the study.

Conclusion. Creatine supplementation at a rate of 2 g/day does not further decrease tHcy concentrations in chronic dialysis patients already treated with high dose folic acid, vitamin B6, and B12 supplementation.

Introduction

Hyperhomocysteinemia, a raised plasma concentration of the sulfur amino acid homocysteine, is observed in several genetic and acquired disorders. The majority of patients with end-stage renal disease (ESRD) have a moderate degree of hyperhomocysteinemia [1]. Treatment of hyperhomocysteinemia consists of folic acid and vitamin B6, B12 supplements. Folic acid supplementation can reduce plasma tHcy about 30% in uraemic patients, whereas the effect of vitamin B6 and B12 is in general much less pronounced [2, 3]. However, the majority of ESRD patients maintain elevated plasma tHcy concentrations when treated with high dose multivitamin supplementation. Betaine can further decrease post-methionine loading tHcy concentrations by 18%, in addition to folic acid supplementation [4]. Additional tHcy-lowering therapies are necessary in these patients in order to attain normal tHcy concentrations.

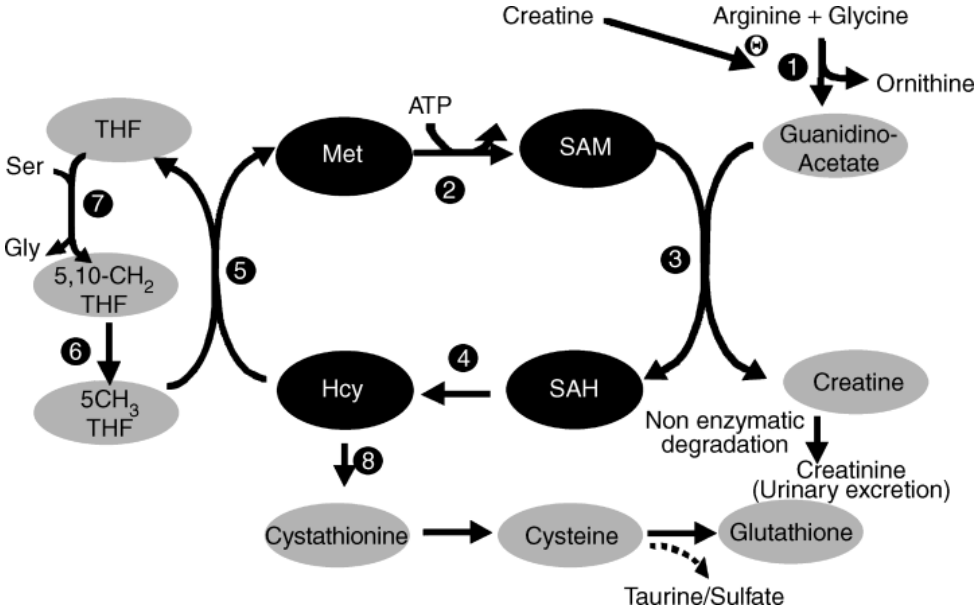

Fig. 1. Overview of homocysteine and creatine metabolism.
THF: tetrahydrofolate; $5CH_3$-THF: 5-methyltetrahydrofolate; 5,10-CH2-THF: 5,10 methylene tetrahydrofolate ; Ser: Serine ; Gly: Glycine; SAM: S-Adenosyl Methionine ; SAH: S-Adenosyl Homocysteine ; Hcy: Homocysteine; Met: Methionine. **Enzymes**: (1) Arginine:Glycine amidinotransferase (AGAT); (2) Methionine Adenosyltransferase ; (3) Guanidinoacetate methyltransferase (GAMT) ; (4) S-Adenosyl Homocysteine hydrolase ; (5) Methionine Synthase ; (6) 5,10-CH2-THF reductase ; (7) Serine-hydroxy methyltransferase ; (8) Cystathionine-β – synthase .

Creatine synthesis and homocysteine (Hcy)-formation are metabolically connected (Fig. 1). Hcy [1, 5] is derived from methionine in a multiple step metabolic cycle (Fig. 1). Methionine can intracellularly be converted to S-adenosyl methionine, a sulfonium compound with a highly reactive methyl group, which acts as a universal methyl donor in numerous transmethylation reactions in vivo. The demethylated product S-adenosyl-homocysteine, a thioether, is readily hydrolyzed to Hcy and adenosine by S-adenosyl-homocysteine hydrolase. Transsulfuration of homocysteine to yield cystathionine is favored in conditions with methionine excess. Remethylation of homocysteine to methionine occurs in conditions of low methionine intake through methyldonation from 5-methyltetrahydrofolate (5-CH_3THF) by means of methionine synthase. Creatine is synthesized in humans by two successive metabolic steps (Fig. 1). Guanidinoacetate is synthesized from glycine and arginine by arginine: glycine aminidinotransferase (AGAT; EC 2.1.4.1), mainly in the kidney. Second, guanidinoacetate is methylated in the liver to creatine by guanidinoacetate-methyltransferase (GAMT) (EC 2.1.1.2) with S-adenosylmethionine as methyl-donor [6,7]. Dietary intake and endogenous synthesis in liver compensate for a daily loss as creatinine of about 2%. Creatine supplementation represses AGAT biosynthesis and, consequently, guanidinoacetate and creatine formation (Fig. 1). Methylation of guanidinoacetate during creatine biosynthesis has been estimated to account for up to 70% of the transmethylation reactions in the body with formation of Hcy [8, 9]. Exogenous creatine supplementation can, thus, be expected to decrease endogenous Hcy synthesis. Inhibition of the methyltransferase reactions has been described in uraemia due to a reduced S-adenosylmethionine (SAM)/S-adenosylhomocysteine (SAH) ratio, and protein hypomethylation and reduced protein repair are observed in uraemic subjects [10, 11]. The influence of creatine on tHcy concentrations, SAM/SAH ratios, and the methyltransferase reactions in humans is currently unknown. Creatine supplementation could reduce the guanidinoacetate-methyltransferase reaction and consequent Hcy-formation. In an animal model of chronic renal failure we have recently demonstrated a pronounced beneficial effect of creatine supplementation on plasma tHcy concentrations. tHcy concentrations were lowered by 22% in creatine-treated animals compared to control diet-fed uraemic animals [12]. Whether creatine can lower tHcy in humans as observed in our animal model is currently unknown.

The present study investigates the effect of creatine supplementation on plasma tHcy concentrations in chronic hemodialysis patients already treated with high-dose multivitamin supplementation.

Methods

Study design and population

Forty-nine hemodialysis patients were recruited. Dialysis was performed with a low-flux triacetate dialyzer (Sureflux-L; Nipro, Osaka, Japan) for 4 to 5 hours 3 times weekly for at least 3 months. Exclusion criteria were: acute illness, life expectancy <3 months, low compliance due to cognitive, social, or psychiatric problems, and inability to provide informed consent. All patients were treated with folic acid 5 mg, pyridoxine 50 mg, and vitamin B12 12 µg orally 3 times a week for at least 3 months. Written informed consent was obtained from all participants. The study protocol was approved by the Ethical Committee of AZ Sint-Jan AV. Sample size estimation (type I error = 0.05; type II error = 0.20), based on the Hcy lowering in our animal study (tHcy = 20%) and tHcy concentrations in dialysis patients, revealed sufficient statistical power of the present study.

The study followed a double-blind, placebo-controlled, crossover design (Fig. 2). Patients received 2 g creatine or placebo daily in the evening during 2 treatment periods of 4 weeks, in random order, and separated by a washout period of 4 weeks. Creatine monohydrate (CreaPure®) was obtained from Degussa Bioactives (Freising, Germany). Placebo tablets contained Fast Flo lactose (Foremost Farms, Baraboo, WI, USA). An exact number of tablets was supplied, and pills were counted to ascertain compliance. Treatment duration, blood flow, dialysate flow, and membrane surface area were determined by the attending nephrologists, but no changes were made during the study period. Single pool Kt/V_{urea} was calculated at the start and end of the study, where K = dialyzer urea clearance, T = duration of dialysis, and V = urea distribution volume at the end of dialysis.

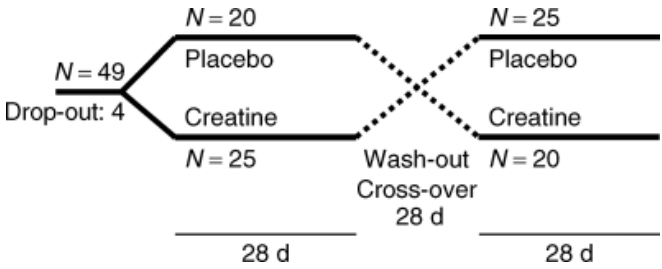

Fig. 2. Treatment schedule

Predialysis blood samples were obtained at baseline and after 4 weeks in both treatment periods. EDTA-samples for tHcy determinations were transported on ice, centrifuged immediately, and stored at -20°C until testing.

Biochemical determinations

Plasma creatinine concentrations were determined using a compensated rate-blanked Jaffé based method (Roche Diagnostics, Mannheim, Germany) on a Modular P analyzer (Roche Diagnostics) according to the manufacturer's procedure. Plasma urea concentrations and aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), alkaline phosphatase, creatine kinase, and γ-glutamyl transferase (GGT) activities were determined on a Modular P analyzer using commercial reagents (Roche Diagnostics).

Total plasma homocysteine concentrations were determined using a fluorescence polarization immunoassay on an Axsym analyzer (Abbott Laboratories, Abbott Park, IL, USA).

Plasma folate and vitamin B12 concentrations were determined using an electrochemiluminescence assay (Roche Diagnostics) on an Elecsys 2010 analyzer (Roche Diagnostics). Plasma and erythrocyte creatine concentrations were determined enzymatically as described before [13]. Plasma creatine was determined without deproteinization, whereas erythrocyte creatine was determined after deproteinization with 5-sulfosalicylic acid. No analytical interference of creatine on tHcy was observed. The prognostic inflammatory and nutritional index (PINI) was calculated as PINI =[(1-acid glycoprotein (mg/L) CRP (mg/L)]/[(albumin (g/L) prealbumin (mg/L)][14, 15]. 1-acid glycoprotein, albumin, and prealbumin were determined nephelometrically using commercial reagents (Dade Behring, Marburg, Germany) on a BN II nephelometer. CRP was determined turbidimetrically on a Modular P analyzer (Roche Diagnostics).

Statistics

Data are expressed as mean ± SD, unless parameters were not normally distributed [median (interquartile range)]. Student t test was used to compare separate groups when appropriate; otherwise nonparametric group comparison was performed using Mann-Whitney U test. Associations between continuous variables were examined using Spearman rank correlation analysis. In order to estimate the effect of creatine treatment on the tHcy concentrations in this crossover framework, a mixed effect model was fitted treating patient as a random effect while the treatment mode, period, and carry-over were modeled as fixed effects [16]. Model

fitting was performed using SAS software (PROC MIXED) (release 8.1, SAS Institute, Inc., Cary, NC, USA). Differences were considered significant at P less than 0.05.

Results

Baseline patient characteristics

Forty-five hemodialysis patients (24 males, 21 females) with a mean age of 70 ± 10 years (range 35-88) were included in this study. Four patients were excluded from the study (2 patients were transplanted during the study, 1 died, and 1 quit the study). Twenty-five patients received creatine, and 20 patients received placebo in the first treatment period. No baseline differences in age, gender distribution, body mass index, tHcy concentrations, or dialysis parameters were observed between placebo and creatine groups for both treatment periods (Table 1).

Table 1. Baseline patient characteristics

	Placebo	Creatine
N	20	25
Male/Female	10/10	14/11
Age *years*	69 ± 12	71 ± 8
Body mass index kg/m^2	24 ± 4	25 ± 3
Plasma creatinine $\mu mol/L$	751 ± 203	698 ± 221
Plasma urea *mmol/L*	22.3 ± 5.7	22.5 ± 6.5
Kt/V_{urea}	1.4 ± 0.4	1.3 ± 0.2

Plasma tHCY and creatine concentrations

No significant changes in plasma tHcy concentrations were observed during treatment with creatine or placebo. Pre- and post-treatment tHcy concentrations were comparable in both creatine or placebo groups during the 2 treatment periods (Fig. 3). Plasma and red cell creatine concentrations were significantly elevated in the creatine treated groups, whereas comparable concentrations were observed before and after placebo treatment, documenting the uptake of creatine (Fig. 3 and Table 3).

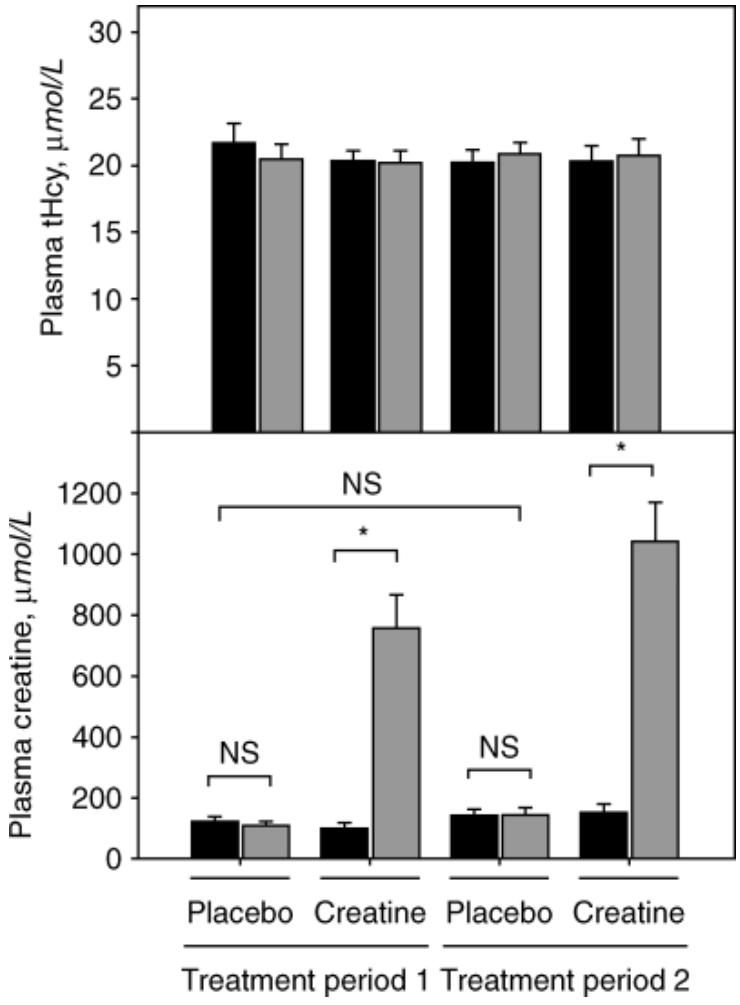

Fig. 3. Plasma homocysteine (tHcy) and creatine concentrations in both treatment periods. Black bars represent concentrations before treatment, whereas gray bars represent concentrations after treatment. Creatine concentrations were significantly higher in the creatine groups, compared to the placebo groups (*: $p<0.0001$). No difference in tHcy concentrations was observed.

	Ref. range	Period 1		Period 2	
		Placebo	Creatine	Placebo	Creatine
Body weight *kg*		68 ± 17	69 ± 10	69 ± 11	68 ± 17
Red cell creatine *mmol/L*		0.30 ± 0.06	0.42 ± 0.11[b]	0.25 ± 0.06	0.39 ± 0.06[b]
Plasma creatinine *μmol/L*	49–103	751 ± 194	884 ± 292[c]	769 ± 230	840 ± 239
Plasma urea *mmol/L*	2.8–8.0	23.3 ± 6.7	25.0 ± 6.7	26.7 ± 5.0	25.0 ± 6.7
AST *U/L*[a]	0–37	17 (15–21)	16 (14–23)	16 (14–22)	16 (15–19)
ALT *U/L*	13–45	15 (11–19)	15 (12–19)	13 (12–19)	14 (11–17)
Alkaline phosphatase *U/L*[a]	56–119	81 (60–103)	92 (75–122)	92 (75–119)	92 (62–120)
GGT *U/L*[a]	11–49	22 (18–26)	21 (14–38)	20 (14–44)	18 (17–27)
Creatine kinase *U/L*[a]	10–195	67 (42–78)	71 (37–99)	60 (33–87)	67 (52–78)
CRP *mg/L*[a]	<5	5 (2–10)	3 (1–8)	3 (1–9)	6 (1–10)
α_1-acid glycoprotein *g/L*	0.5–1.3	1.00 ± 0.25	0.98 ± 0.28	1.01 ± 0.27	1.01 ± 0.26
Albumin *g/L*	34–48	40.1 ± 4.7	39.5 ± 5.6	39.9 ± 4.8	40.0 ± 4.0
Prealbumin *g/L*	0.2–0.4	0.26 ± 0.07	0.28 ± 0.10	0.29 ± 0.08	0.25 ± 0.06
PINI[a]	<1.0	0.47 (0.13–1.0)	0.25 (0.07–0.76)	0.25 (0.09–0.91)	0.42 (0.14–1.3)
Total protein *g/L*	66–83	67 ± 3.9	66 ± 4.5	66 ± 4.4	66 ± 35
Folate *nmol/L*	4.5–20.3	69.9 ± 13.4	63.2 ± 15.6	66.1 ± 19.1	73.0 ± 14.9
Vitamin B12 *pmol/L*	145–639	579 ± 192	483 ± 199	581 ± 190	636 ± 157

Abbreviations are: PINI, prognostic inflammatory and nutritional index; AST, aspartate-aminotransferase; ALT, alanine-aminotransferase; GGT, γ-glutamyl transferase.

[a]Non-Gaussian distribution, data expressed as median (interquartile range).

[b]$P < 0.0001$.

[c]$P < 0.01$, creatine treatment compared to placebo treatment in both treatment periods.

Table 3. Biochemical variables in both treatment groups

No difference in baseline creatine concentrations was observed between the first and second treatment period, documenting the washout effect (Fig. 3). Male patients had significantly higher tHcy concentrations (23.3 ± 3.7 μmol/L) compared to female patients (18.7 ± 6.1 μmol/L; P= 0.005). No effect of creatine supplementation on tHcy concentrations was observed in either male or female patients (data not shown).

The results of the mixed effects model fit for tHcy are shown in Table 2. No treatment, period, or carryover effects were detected. No correlation between plasma creatine and tHcy concentrations was observed (r=0.08; P= 0.76).

Table 2. Results of the mixed effects model fit

	β (SE)	t value	Significance
Treatment (creatine vs. placebo)	−0.253 (0.634)	−0.40	$P = 0.69$
Carryover effect	−0.259 (1.409)	−0.18	$P = 0.86$
Period (2nd vs. 1st)	−0.393 (0.634)	−0.62	$P = 0.54$

Plasma folate and vitamin B12 concentrations

All patients had elevated plasma concentrations of folate and vitamin B12 (Table 3). No difference in plasma folate or vitamin B12 was observed between creatine- or placebo-treated groups. No significant relationship between tHcy and plasma folate or vitamin B12 concentrations was observed.

Nutritional status and dialysis

Nutritional status remained constant during the study period. No difference over time in total protein, albumin, prealbumin concentrations, and PINI scores were observed in any of the groups. A significant relationship between total protein concentration and tHcy was observed (r= 0.20; P= 0.006). No relationship between albumin (r= 0.103; P= 0.17), prealbumin (r= 0.10; P= 0.20) concentrations, or PINI scores (r= 0.01; P= 0.87) and tHcy was observed in this population. Body weight and Kt/V_{urea} remained constant during the study period. No difference in Kt/V_{urea} between creatine- and placebo-treated groups [1.3 ± 0.3 vs. 1.4 ± 0.3 (post-treatment)] was observed. Baseline tHcy concentrations correlated with Kt/V_{urea} (r=0.35; P= 0.01) and plasma creatinine concentrations (r= 0.40;

P= 0.004) (Fig. 4). No relationship after treatment was observed between Kt/Vurea and tHcy concentrations.

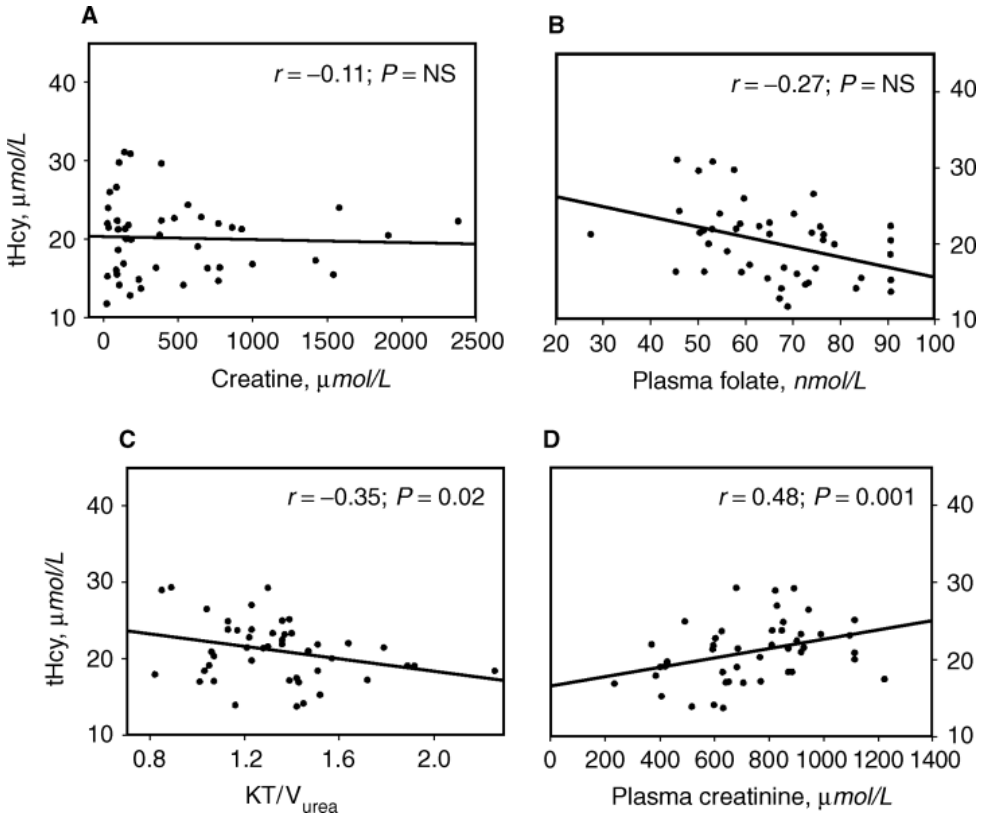

Fig. 4. Relationship between plasma homocysteine (tHcy) and plasma creatine (A), plasma folate (B), KT/V_{urea} (C) and plasma creatinine concentrations (D). Panel A, B represent values (homocysteine, creatine, folate) after treatment; panel C, D represent baseline values (homocysteine, creatinine, KT/V_{urea}).

Discussion

Hcy metabolism is tightly regulated under normal conditions. However, in situations with compromised Hcy homeostasis such as dialysis, creatine supplementation could have a more prominent tHcy-lowering effect [17]. In our dialysis patients treated with folic acid, vitamin B6, and B12, plasma tHcy concentrations remained moderately elevated, an observation in agreement with the well-known resistance to therapy in uraemia. In contrast to the effects in experimental animals [12, 18], oral creatine supplementation at a rate of 2 g/day did not lower plasma tHcy

concentrations in our dialysis population. tHcy concentrations were comparable after treatment with creatine and placebo. This observation is in line with the absence of an effect of creatine supplementation on tHcy in young healthy volunteers [19].

Plasma creatine and creatinine concentrations were higher in creatine-supplemented subjects, documenting uptake and metabolization of creatine. The absence of effect could, thus, not be attributed to a limited uptake of creatine. In addition, the sample size was sufficiently large to demonstrate a decrease in plasma tHcy as observed previously in our animal study [12]. The dose of 2g creatine daily was chosen on the basis of existing treatment schedules. In healthy subjects, creatine at a maintenance dose of 2 g is used as a performance enhancing nutritional supplement, with documented uptake of creatine in the skeletal muscle [20]. Loading doses up to 20 g/day are used by athletes. We have chosen not to use a loading dose to avoid extremely high plasma concentrations with potential side effects.

In contrast to our experimental animals, our patient population was treated with a chronic high-dose multivitamin regimen. High-dose folic acid supplementation could mask the Hcy-lowering effect of creatine in this population. A clinically relevant tHcy-lowering treatment should be able to decrease tHcy levels in vitamin-replete patients. Theoretically, lowering methylation demand could be synergistic with folic acid supplementation, which enhances the remethylation pathway. In contrast to these theoretic expectations, a decreased methylation demand by exogenous creatine supplementation did not lower tHcy concentrations, despite documented uptake and metabolization of creatine.

In our patient population, creatine supplementation did not alter plasma folate concentrations. High-dose folic acid supplementation could have masked the folate sparing action of creatine that we have observed in our animal model. Lowering methyl group consumption by creatine supplementation apparently no longer influences tHcy or folate concentrations in conditions of excess folate [12].

Species-related differences in metabolic pathways could perhaps explain the difference in effect between humans and rodents. In rats, about 75% of plasma tHcy was found to be free, whereas in humans, about 65% to 75% of tHcy is bound to protein by a disulfide bond [21, 22]. In dialysis patients, protein binding of Hcy was even found to be higher in comparison to nondialyzed chronic uraemic patients [23]. These differences in protein binding could be reflected in differences found in renal extraction of Hcy in humans and rats. In rats, significant clearance of Hcy by the kidneys was observed [22], whereas in humans, no arteriovenous difference in Hcy

concentrations was observed [24]. Differences in renal handling and in general Hcy metabolism could account for the absence of effect of creatine supplementation in our study. Aside from dissimilarities in Hcy-metabolism, creatine could be metabolized differently in rats than in humans. Compartmentalization and possibly regulation of the different enzymes necessary for creatine biosynthesis differs substantially between species [6, 25].

In uraemia, a disturbed SAM/SAH ratio inhibits the methyltransferase reactions. Creatine supplementation has been described to reduce the guanidinoacetate-methyltransferase reaction by lowering guanidinoacetate concentrations. The influence of creatine on other methyltransferase reactions (e.g., DNA-, protein methyltransferases) is currently unknown. Because creatine supplementation fails to lower tHcy concentrations in our dialysis population, the effect on other methyltransferase reactions will probably be limited in humans.

The safety of creatine supplementation and the absence of adverse effects on renal function were previously assessed by our group in an animal model of chronic renal failure [26]. No adverse effects on glomerular filtration or renal protein handling were observed. Although adverse effects of creatine have been suggested, none of the controlled studies in humans on the effects of creatine supplementation have revealed any [27]. In our hemodialysis population we did not observe any major adverse effect of creatine administration. Plasma creatinine concentrations were slightly elevated due to increased creatine load in the creatine groups compared to the placebo groups. No effect on body weight or liver enzymes was noted.

Protein malnutrition is common in patients on maintenance dialysis. Malnutrition and hypoalbuminemia are risk factors for increased morbidity and mortality in patients with ESRD [28, 29]. Nutritional status influences plasma tHcy concentrations by the strong protein binding of Hcy to albumin, and by the increased Hcy formation upon methionine uptake from the diet. The PINI is a formula to evaluate nutritional status and prognosis in critically ill patients [14, 15]. The PINI score has been measured in several settings, and has been found to be a reliable indicator of both nutritional status and prognosis. The PINI score was determined in this study in order to take into account the effect of protein malnutrition on tHcy concentrations. In our population we evaluated the relationship between nutritional status by means of the PINI score and tHcy concentrations, as tHcy depend greatly on protein intake [30, 31]. During the study no changes in nutritional status were observed in either creatine- or placebo-treated group. We observed no correlation between PINI scores, albumin or prealbumin concentrations, and tHcy concentrations. In

conditions of excess folate, nutritional status could be of less importance in determining plasma tHcy concentrations.

Creatine supplementation has been shown to exert ergogenic effects in several populations. In dialysis patients, creatine supplementation was shown to decrease muscle cramps during dialysis [32]. In other conditions associated with low muscular mass and performance [33], creatine was shown to increase muscular performance and quality of life. Further research is necessary to evaluate the potential use and mechanisms of creatine as an ergogenic substance in dialysis patients.

Conclusion

The present study demonstrated that inhibition of endogenous methylation demand with dietary creatine supplementation does not further decrease tHcy concentrations in chronic dialysis patients treated with high-dose folic acid, vitamin B6, and B12 supplementation.

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Note added to manuscript in thesis:

As stated in the introduction of this thesis, the relation between tHcy and cardiovascular risk remains unknown and several epidemiological studies could not demonstrate an effect of tHcy-lowering therapy on cardiovascular mortality. Till today, the importance of Hcy in the pathogenesis of atherosclerosis remains elusive (Suliman, 2005). In this regard, the statements about tHcy-lowering therapy in this manuscript should be considered in this perspective.

This study was carried out in a chronic hemodialysis population, intermittently treated with erythropoietin (EPO). Theoretically EPO-treatment could have influenced the red cell creatine content, as EPO enhances the red cell creatine concentrations (Schmidt et al., 1990). The red cell creatine concentrations in this study were determined to confirm the creatine uptake in the body and not as a marker for muscular creatine

concentrations. Recent evidence points out that the red cell creatine content is a poor marker for the muscular creatine content and should therefore not be used as a surrogate marker (Preen et al., 2005).

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CHAPTER 3.3

Effect of creatine supplementation on muscular performance in aging men and muscle metabolism in rats

Effects of creatine supplementation and exercise training on fitness in men 55–75 yr old.
J Appl Physiol 2003;95:818–828

Effect of dietary creatine on skeletal muscle myosin heavy chain isoform expression in an animal model of uraemia.
Nephron Exp Nephrol 2004;96:e103-110

Effects of creatine supplementation and exercise training on fitness in men 55–75 yr old

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Abstract

The effect of oral creatine supplementation (CR; 5 g/day) in conjunction with exercise training on physical fitness was investigated in men between 55 and 75 yr of age (n = 46). A double-blind randomized placebo-controlled (PL) trial was performed over a 6-mo period. Furthermore, a subgroup (n = 20) completed a 1-yr follow-up. The training program consisted of cardiorespiratory endurance training as well as moderate resistance training (2–3 sessions/wk). Endurance capacity was evaluated during a maximal incremental bicycle ergometer test, maximal isometric strength of the knee-extensor muscles was assessed by an isokinetic dynamometer, and body composition was assessed by hydrostatic weighing. Furthermore, in a subgroup (PL: n = 13; CR: n = 12) biopsies were taken from m. vastus lateralis to determine total creatine (TCr) content. In PL, 6 mo of training increased peak oxygen uptake rate (+16%; $P < 0.05$). Fat-free mass slightly increased (+0.3 kg; $P < 0.05$), whereas percent body fat slightly decreased (-1.2%; $P < 0.05$). The training intervention did not significantly change either maximal isometric strength or body weight. The responses were independent of CR. Still, compared with PL, TCr was increased by 5% in CR, and this increase was closely correlated with initial muscle creatine content ($r = -0.78$; $P < 0.05$). After a 1-yr follow-up, muscle TCr was not higher in CR than in PL. Furthermore, the other measurements were not affected by CR. It is concluded that long-term creatine intake (5 g/day) in conjunction with exercise training does not beneficially impact physical fitness in men between 55 and 75 yr of age.

Introduction

A primary strategy to optimize health in older people is to prevent potential medical problems from reaching an overt clinical state (29). In this respect, research over the last 20–30 yr has clearly shown that increasing the level of physical activity is an effective intervention to alleviate the normal deterioration of health-related fitness parameters (35), which include functional capacity of the musculoskeletal and cardiovascular system, body composition, and metabolic health (8). Resistance training can reduce the pace of age-related muscle atrophy and the concomitant decline of muscular functional capacity (19, 21, 28). The latter is very critical to maintain the ability to perform activities of daily living. Furthermore, endurance exercise training can enhance cardiorespiratory fitness in older people (14). Endurance training also reduces cardiovascular risk by beneficially impacting cardiovascular risk factors such as adverse blood lipid profile, high blood pressure, and impaired glucose tolerance due to peripheral insulin resistance (29, 50). Thus published literature provides strong evidence to suggest that both aerobic endurance and resistance training can beneficially impact physical fitness in older people.

Another intervention that might contribute to enhance physical fitness in older people is oral creatine supplementation (CR). The potential of oral creatine (Cr) intake to increase muscle Cr content, and thereby enhance muscular performance during short maximal exercise over repeated bouts in the young, has been extensively documented over the last 10 yr (47, 53). The finding that CR can stimulate the beneficial effects of resistance training on muscle volume and functional capacity, at least in young healthy subjects (5, 27, 34, 48, 50), is interesting in the context of the preservation of muscle functional capacity in the elderly. Furthermore, consistent with the observations in young healthy subjects, CR has been found to enhance muscular functional capacity in patients afflicted by neuromuscular diseases (45, 51, 52) as well as in cardiac patients (11, 22). It has been suggested that older people might respond better to CR than young people, because they may have a lower muscle Cr content (46). However, literature data are not consistent with regard to the latter issue (12, 31, 36, 37), and a recent study (41) has even shown Cr content in muscles from older people to be higher than in younger people. Furthermore, some earlier studies have found the effects of CR in conjunction with resistance training on muscular performance capacity in the elderly to be either absent (7, 42), small (11), or moderate (10). However, the intervention periods in the latter studies were short (7 days to 14 wk) relative to the need for long-term physical activity in the older population. Moreover, the training interventions used in the above studies involved only heavy resistance training. However, in older persons, a mixed training program combining both cardiorespiratory stimulation and moderate resistance training probably is more optimal than selective high-

load weight training (3, 29). Therefore, to improve our understanding of the efficacy of Cr intake as a potential ergogenic aid in the older population, it is warranted to evaluate the effects of long-term Cr intake in conjunction with an exercise training program involving both endurance training and moderate resistance training (47).

Long-term Cr intake currently is being promoted as a health-enhancing substance. At present, however, no study has investigated the impact of long-term (12 mo) low dose (5 g/day) Cr intake on a variety of safety parameters. Because knowledge regarding the occurrence of adverse side effects after the supplementary intake of any "ergogenic" substance is essential, it is necessary to investigate this matter. Furthermore, some (1, 17), but not all (49), studies have reported CR in combination with resistance training to have a cholesterol-lowering effect. Because of the high prevalence of elevated blood cholesterol at older age, it is therefore warranted to further investigate the potential cholesterol-lowering effect after long-term Cr intake.

Therefore, the purpose of the present study was to evaluate whether long-term Cr intake in conjunction with exercise training in older men is safe and can have a beneficial impact on physical fitness, including muscle strength and cardiorespiratory endurance.

Methods

Subjects

Subjects were recruited from the staff (active or retired) at the local university. Men older than 55 yr were invited to participate in a meeting aimed to explain the purpose and details of the study protocol. Within the next week, 57 subjects, who met the inclusion criteria, volunteered to participate in the study. After being informed of all the experimental procedures to be undertaken, they gave their written, informed consent and were enrolled in the study. Inclusion criteria were 1) men between 55 and 75 yr of age; 2) no participation in strength training for at least 5 yr; 3) participation in low-intensity physical activity, such as gardening and walking, for <4 h/wk; and 4) no history of oral Cr intake. All volunteers underwent a preliminary clinical examination and were submitted to an electrocardiogram (ECG)-controlled maximal exercise test. Exclusion criteria on admission were prehistory of kidney disease, albuminuria, consistent intake of any medication known to impair exercise capacity, and any disease contraindicating high-intensity exercise training. From the 57 volunteers screened, 11 were excluded because of a significant ST depression (1.5 mm or more; n = 4), complicated ventricular arrhythmias (n = 3), arterial fibrillation (n = 1), hypertension (n = 2), and ECG-diagnosed posterior myocardial infarction (n = 1). Subjects were asked to

avoid changes in their diet and level of physical activity (except for the fitness training program prescribed by the study protocol), and to maintain constant living habits during the period of the study.

Study Protocol

The local ethics committee approved the study protocol. A double-blind study was performed over a 1-yr period that involved two phases (phase I: 0–6 mo; phase II: 6 mo to 1 yr). Baseline measurements were performed on 3 separate days, each separated by a 2-day interval. On day 1, after a standardized warm-up, subjects performed an exercise test on an isokinetic dynamometer to evaluate maximal torque and fatigue of the knee extensor muscles. Test-retest reliability for these torque measurements was 0.93 (intraclass correlation [2,1]). Immediately afterward, body composition was assessed in the seated position by hydrostatic weighing. On day 2, subjects reported to the laboratory for a maximal incremental exercise test on an electromagnetically braked bicycle ergometer. Finally, on day 3, the subjects reported to the laboratory in the morning after an overnight fast. A blood sample was taken from an antecubital vein into heparinized tubes (Vacutainer) for clinical biochemistry. Furthermore, in a subgroup of subjects, a muscle biopsy was taken from the vastus lateralis muscle under local anesthesia for biochemical and histochemical analysis on day 4. After the baseline measurements, subjects were coupled into pairs that were matched for age, maximal isometric knee-extension torque, and peak oxygen uptake (O_2peak). Thereafter, each pair was assigned, in a double-blind manner and by an independent investigator who was otherwise not involved in the study, to either a PL (n = 23; age: 62.2 ± 1.3 yr: body weight: 81.3 ± 2.5 kg) or a CR (n = 23; age: 63.9 ± 1.1 yr; body weight: 79.2 ± 2.3 kg) group.

After baseline measurements, subjects were enrolled in a well-controlled and supervised fitness training program. The training-program aimed to increase cardiorespiratory fitness as well as enhance strength of the abdominal and back muscles and the primary peripheral muscle groups. During phase I, subjects were instructed to participate in 10 training sessions per 4-wk "window." To ensure adequate recovery between training sessions, training sessions were interspersed by at least 1 rest day. Each training session (75 min) started with two endurance exercise bouts, which first involved bicycle ergometry (12 min) and thereafter treadmill walking/jogging or rowing ergometry (12 min). To monitor the training workload, exercise intensity was controlled by heart rate monitoring. Exercise intensity was initially set at 65% of the individual heart rate reserve (Karvonen formula) and was increased gradually to 80% of heart rate reserve toward the end of the training period. The bicycle ergometers and treadmills used (Technogym) automatically estimated the

number of calories spent during the exercise performed. These values were noted in the individual training diary. For the rowing ergometry, resistance was fixed and the estimated distance covered was noted in a training diary. The latter values were later used as a global measure to compare the amount of endurance work performed between groups. After the endurance training bouts, the subjects started a moderate-resistance weight training session. They performed seven exercises (sit-ups, arm curl, back extension, leg extension, leg press, vertical row, and lateral pull on Technogym gym apparatus). Each exercise consisted of two series of 30 repetitions at 30-repetition maximum (RM) workload. To monitor training workload, subjects were instructed to note the resistance used (in kg) in their training diary for the two series of each exercise.

From the start of the training program, CR received 5 g of Cr monohydrate tablets per day, whereas the placebo-controlled (PL) group received placebo tablets. On nontraining days, subjects ingested one tablet before breakfast, three before lunch, and one before dinner. However, on training days, the 3-g dose was to be ingested immediately after the training session. Hence, before lunch and dinner on morning training days, and before breakfast and lunch on evening training days, subjects ingested one tablet. After 3 and 6 mo, and at least 48 h after their last training session, subjects returned to the laboratory to participate in the same measurements as at baseline. However, at 3 mo, no muscle biopsy was taken. At each occasion, the tests were performed at the same time of day and by the same investigator. At the end of phase I, subjects were asked to either engage for an additional 6 mo of follow-up (phase II) or to withdraw from the study. Thirty-six subjects agreed to continue participating in the study (PL: n = 21, age: 61.8 ± 1.3 yr; CR: n = 15, age: 65.3 ± 1.3 yr) and 10 withdrew (PL: n = 2; CR: n = 8). Thus, in phase II of this study, subjects were not randomly selected but instead voluntarily chose to remain in the study. To enhance training compliance on the one hand and to meet the expectations by the subjects on the fitness training on the other hand, the training protocol was slightly adapted. Minimum training rate was reduced from 10 to 8 sessions per 4-wk window. Furthermore, every third training session workload for the weight-lifting exercises was increased from 30 to 20 RM. Finally, the duration of the endurance exercise bouts was increased from 12 to 15 min. Otherwise the study protocol was identical to phase I of the study. After 12 mo, subjects returned to the laboratory to participate in the same measurements as at baseline. However, muscle biopsies were only taken at the end of the study and only in the subjects who had been biopsied in phase I. At each occasion (phase I and phase II), tests were performed at the same time of day and by the same investigator. None of the study results were disclosed to either the subjects or to the investigators until the end of the entire study.

Maximal Exercise Testing and Spirometry

The maximal exercise tests on an electromagnetically braked bicycle ergometer (Ergometrics model 800S, Bitz, Germany) were performed in a laboratory where room temperature was stabilized at 18–22°C. At the occasion of the first test, seat height was noted and reproduced for all subsequent tests. Subjects first rested for 10 min in the upright seated position on the ergometer, where their blood pressure was measured by using an automated sphygmomanometer (model STBP-780, Colin, Komaki, Japan). Thereafter, the exercise test was started at an initial workload of 20 W, which was increased by 20 W every minute until volitional exhaustion. During the test, a 12-lead ECG (Marquette, WI) was continuously monitored. Furthermore, ventilation and oxygen and carbon dioxide concentrations in the inspired and expired air were continuously measured by a calibrated breath-by-breath system (Oxycon Alpha, Jaeger-Mijnhardt, Bunnik, The Netherlands) and oxygen uptake (O_2), carbon dioxide output (CO_2), and the respiratory gas exchange ratio (RER; CO_2/ O_2) were automatically calculated. The V_{slope} threshold, to be used as an estimate of the "anaerobic threshold" in the context of this manuscript, was determined as the exercise intensity corresponding with the transition of the RER relationship from a linear to an exponential curve (4).

Determination of Maximal Dynamic Knee-Extension and Isometric Force

Maximal voluntary torque and power of the knee extensors was evaluated on an isokinetic dynamometer that consisted of a computer-controlled asynchronous electromotor (AMK Dynasyn, 19 kW), instrumented with a torque transducer (Lebow, maximal torque 565 N•m, 0.05% precision). The exercise test consisted of unilateral knee extensions performed in a semisupine sitting position on the dynamometer. After a 5-min standardized warm-up, the subjects performed two voluntary maximal isometric contractions (3 s), interspersed by 2-min rest intervals, at knee angles of 90, 110, and 130°, respectively. Maximal isometric torque (in N•m) was obtained from the smoothed curve of the static torque and was calculated as the average of the three knee angles (90, 110, and 130°). Thereafter, subjects performed two bouts of 30 dynamic maximal voluntary knee extensions, interspersed by a 2-min rest interval, at a constant velocity of 180°/s, starting from 90° to full extension (180°). After each contraction, the leg was returned (180°/s) passively to the starting position from which the next contraction was immediately initiated. Torque and angular velocity were measured during each contraction and were simultaneously digitized (250 Hz) by an on-line computer.

Body Composition

Body composition was assessed in the seated position by hydrostatic weighing. Residual lung volume was measured by the helium-dilution

technique, and gastrointestinal tract air volume was assumed to be 150 ml. Body density was converted to percent body fat by using Siri's equation (43), where percent body fat is equal to [4.95/(density - 4.5)] x 100.

Muscle Biochemistry and Histochemistry

Muscle samples were obtained from the vastus lateralis muscle of the right leg by using the needle biopsy technique. Incisions were made through the skin and muscle fascia after the administration of 2–3 ml of local anesthesia (lidocaine, 1%). After removal from the limb, a piece of each muscle biopsy was immediately freed from blood and visible connective tissue, rapidly frozen in liquid nitrogen, and stored at -80°C for subsequent biochemical analysis. The remaining muscle was mounted in embedding medium, frozen in isopentane, cooled to its freezing point in liquid nitrogen, and stored at -80°C until analyses were performed at a later date. For muscle substrate assays, muscle samples were freeze-dried. Thereafter, a portion (3–5 mg) of each sample was dissected free of visible blood and connective tissue. Muscle ATP, free Cr, and phosphocreatine (PCr) contents were analyzed from perchloric acid precipitated extractions by using standard fluorometric assays (6). Because muscle ATP content normally does not change as a result of the interventions or treatments used, free Cr and PCr content was corrected for the individual mean ATP content over the time points. Total Cr (TCr) content was calculated by summing free Cr and PCr content. For the histochemical analyses, serial transverse sections (10 μm) were cut from the biopsies with a microtome at -20°C and stained for myofibrillar ATPase to identify fiber types (9).

Clinical Chemistry

Routine blood and urine clinical screening tests were performed during the course of the study. The serum samples were immediately transferred to a local routine clinical biochemistry laboratory for determination of white and red blood cells, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, and red cell distribution width by using a Beckman-Coulter autoanalyzer, and of glutamate oxalate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, Cr kinase, urea, and urate by using a Hitachi autoanalyzer both running on Roche diagnostic reagents. All plasma samples and a fraction of the 24-h urine samples were stored at -20°C to be analyzed at the end of the study. Plasma and urinary Cr concentrations were measured by a standard enzymatic fluorometric assay (6). Plasma and urinary creatinine concentration were assayed by a rate-blanked kinetic Jaffé-based method (alkalic picrate, Roche Diagnostics).

In the subgroup that completed the 1-yr follow-up, we also measured blood lipids. Total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were analyzed using a cholesterol oxidase-based method, after which lowdensity lipoprotein (LDL) cholesterol was calculated

according to the following formula: total cholesterol - HDL-cholesterol - (triglicerides/5).

Statistical Analyses

Separate statistical analyses were performed on the data in the total group of subjects (phase I, 6-mo follow-up; n = 46) and on the subgroup that also participated in phase II of the study (phase I and II, 1-yr follow-up; n = 20). Missing data (5% for the muscle histochemistry and blood biochemistry data; 2% for all other data) were imputed according to the null hypothesis. Phase I data were analyzed according to the "intention-to-treat" principle. Phase I and II data were analyzed according to the "as-treated" principle. Treatment effects were evaluated by using two-way analyses of variance that were covariate adjusted for the baseline value (Statistica, Statsoft, Tulsa, OK). When appropriate, Tukey's post hoc tests were applied. In addition, we also performed a one-way analysis of variance to compare within the groups the baseline values with the values obtained after 3, 6, and 12 mo of follow-up. The relationship between variables was calculated by using Pearson's correlation coefficient. Statistical significance was taken at a probability level of $P < 0.05$. All data are expressed as means ± SE.

Results

Training Compliance

Six-month follow-up. The number of training sessions completed was similar between PL (56 ± 3 training sessions) and CR (59 ± 2 training sessions). Furthermore, the total number of calories spent in walking and/or running (PL: 4,420 ± 370 kcal; CR: 4,340 ± 210 kcal) and bicycling (PL: 6,880 ± 410 kcal; CR: 6,560 ± 280 kcal), as well as the total estimated distance covered during rowing ergometry (PL: 56 ± 5 km; CR: 58 ± 4 km) did not differ between groups. Workloads for leg extension and arm curl, the two exercises that were obligatory throughout the training period, were similar between PL and CR both during the initial 4 wk of the 6-mo training period (PL leg extension: 27 ± 2 kg, arm curl: 16 ± 1 kg; CR leg extension: 29 ± 2 kg, arm curl: 16 ± 1 kg), increasing to the period (PL leg extension: 49 ± 5 kg, arm curl: 27 ± 2 kg; CR leg extension: 49 ± 5 kg, arm curl: 26 ± 2 kg) during the final 4 wk ($P < 0.05$).

One-year follow-up. The number of training sessions completed during phase I was 67 ± 2 in both PL and CR. Furthermore, the number of calories spent in walking and/or running (PL: 5,160 ± 570 kcal; CR: 4,530 ± 260 kcal) and bicycling (PL: 8,200 ± 520 kcal; CR: 7,490 ± 320 kcal), and the distance covered during rowing ergometry (PL: 68 ± 9 km; CR: 69 ± 5 km) did not vary between groups. During phase II of the study,

minimum training rate was reduced from 10 to 8 sessions per 4-wk training cycle. Hence, compared with phase I, the number of training sessions completed decreased (PL: 41 ± 3 training sessions; CR: 46 ± 2 training sessions). Compared with PL (3,020 ± 420 kcal), the number of calories spent in walking and/or running was higher ($P < 0.05$) in CR (3,940 ± 280 kcal). However, neither the number of calories spent in cycling (PL: 6,220 ± 600 kcal; CR: 6,430 ± 530 kcal) nor the distance covered during rowing (PL: 59 ± 7km; CR: 56 ± 6 km) was different between PL and CR. Workloads for leg extension and arm curl during the first 4-wk training period were similar in PL (leg extension: 28 ± 3 kg; arm curl: 17 ± 2 kg) and CR (leg extension: 31 ± 3 kg; arm curl: 16 ± 1 kg). Six months of training increased leg extension and arm curl workloads by 75% in either group. At month 12, compared with baseline, leg extension workload was increased by another 15% in both PL and CR ($P < 0.05$), whereas arm curl workload was decreased by 20% in both groups ($P < 0.05$).

Side Effects

All subjects underwent cardiological screening, including stress ECG testing, before the start of the study. Still, during phase I of the study, five subjects (4 in CR vs. 1 in PL; P value not significatnt) developed significant ST depression at the occasion of the exercise test at either month 3 ($n = 3$) or month 6 ($n = 2$). Three of these subjects were immediately treated by coronary balloon dilatation (percutaneous transluminal coronary angioplasty). Thereafter, they participated in a cardiac rehabilitation exercise program for 1 mo and eventually resumed the experimental training program. Within 1 mo after resuming the training program, they reached the same intensity level as before treatment, yet they did not exhibit ST depression in any later exercise test. The two other subjects were instructed to quit the training study and were enrolled in a cardiovascular rehabilitation program after having received the appropriate treatment. Furthermore, during the study, one subject of the CR group was afflicted from overuse trauma at the level of the left shoulder joint. This subject continued to follow the exercise training program except for the weight-lifting exercises involving shoulder activity. However, after appropriate physiotherapy (6 wk), this subject also resumed the latter part of the training program.

Cardiorespiratory Measurements

Six-month follow-up. At the start of the study, O_2, workload, heart rate, and RER at V_{slope} threshold (VT) intensity as well as at exhaustion (peak) were similar between groups (Table 1). In PL, 3 and 6 mo of training increased O_2 at VT intensity by 27 and 19%, respectively ($P < 0.05$). At peak intensity, corresponding increases in O_2peak were 13 and 16% ($P < 0.05$). Compared with baseline, workload at VT was increased by 16%

after 3 mo of training ($P < 0.05$) but not after 6 mo [+3%; not significant]. Peak workload was increased by 7 and 11% at 3 and 6 mo, respectively ($P < 0.05$). O_2 and workload corresponding with either VT or peak were not significantly different between PL and CR at any time of the study. Furthermore, heart rate and RER at VT and peak were similar between PL and CR after both 3 and 6 mo of training. At baseline, resting diastolic (PL: 84 ± 2 mmHg; CR: 80 ± 2 mmHg) and systolic blood pressures (PL: 139 ± 3 mmHg; CR: 136 ± 3 mmHg) were similar in PL and CR. Compared with PL (138 ± 5 mmHg), 3 mo of training decreased systolic pressure in CR (125 ± 2 mmHg; $P < 0.05$). However, this difference had disappeared by 6 mo of training. Diastolic blood pressure was not different between the experimental groups at any time of the study.

Table 1. Effect of creatine intake in conjunction with fitness training on cardiorespiratory endurance in men 55-75 yr old

	Baseline		3 mo		6 mo		12 mo	
	Placebo	Creatine	Placebo	Creatine	Placebo	Creatine	Placebo	Creatine
6-mo follow-up (n = 23)								
$\dot{V}o_2$, ml/min								
VT	1,492 ± 78	1,572 ± 109	1,893 ± 81*	1,662 ± 71	1,778 ± 80*	1,806 ± 72		
Peak	2,333 ± 81	2,292 ± 96	2,629 ± 86*	2,497 ± 86*	2,700 ± 94*	2,509 ± 90*		
Workload, W								
VT	126 ± 7	132 ± 9	146 ± 7	128 ± 6	130 ± 7	116 ± 5*		
Peak	210 ± 8	204 ± 7	224 ± 8*	216 ± 7*	235 ± 9*	225 ± 8*		
Heart rate, beats/min								
VT	112 ± 4	118 ± 5	119 ± 3*	117 ± 3	113 ± 3	109 ± 4*		
Peak	148 ± 3	150 ± 5	154 ± 3	151 ± 4	149 ± 3	148 ± 5		
RER								
VT	0.93 ± 0.01	0.96 ± 0.01	0.99 ± 0.01*	0.98 ± 0.01	0.99 ± 0.02*	0.97 ± 0.01		
Peak	1.15 ± 0.02	1.13 ± 0.02	1.17 ± 0.01	1.20 ± 0.01*	1.19 ± 0.01*	1.21 ± 0.02*		
1-yr follow-up (n = 10)								
$\dot{V}o_2$ ml/min								
VT	1,577 ± 132	1,699 ± 160	1,962 ± 133*	1,704 ± 125	1,873 ± 149*	1,591 ± 118	1,697 ± 107	1,578 ± 116
Peak	2,330 ± 134	2,201 ± 80	2,631 ± 139*	2,472 ± 60*	2,742 ± 171*	2,521 ± 89*	2,802 ± 178*	2,650 ± 140*
Workload, W								
VT	130 ± 11	142 ± 15	156 ± 12*	134 ± 10	140 ± 11	114 ± 7*	119 ± 9	109 ± 9*
Peak	206 ± 14	204 ± 8	227 ± 16*	224 ± 7*	244 ± 16*	232 ± 9*	224 ± 15	216 ± 9
Heart rate, beats/min								
VT	111 ± 6	120 ± 8	118 ± 5	115 ± 5	109 ± 4	107 ± 5*	108 ± 2.8	112 ± 6
Peak	145 ± 6	147 ± 7	149 ± 5	150 ± 7	148 ± 5	147 ± 7	149 ± 5	152 ± 7
RER								
VT	0.99 ± 0.01	0.97 ± 0.01	0.98 ± 0.01	0.97 ± 0.01	0.93 ± 0.02*	0.98 ± 0.02	0.97 ± 0.01	0.98 ± 0.01
Peak	1.13 ± 0.01	1.13 ± 0.02	1.17 ± 0.01	1.20 ± 0.02	1.18 ± 0.02	1.22 ± 0.03	1.11 ± 0.07	1.10 ± 0.07

Values are means ± SE of 23 and 10 observations for the 6-mo and 1-yr follow-up, respectively. A graded maximal cycle ergometer test was performed before (baseline) and after 3, 6, and 12 mo of fitness training combined with either placebo or creatine intake (5 g/day). Oxygen uptake rate ($\dot{V}o_2$), workload, heart rate, and respiratory gas exchange ratio (RER) were determined at the exercise intensity corresponding with the V_{slope} threshold (VT) and at the point of volitional exhaustion (peak). *$P < 0.05$ compared with the corresponding baseline value. See METHODS for further details.

One-year follow-up. As shown in Table 1, O_2, workload, heart rate, and RER values measured at both VT and peak, at 0, 3, and 6 mo of training, were nearly identical in the total groups of subjects (6-mo followup) and in the subgroup of subjects who completed the 1-yr follow-up. Compared with baseline, in PL at the end of the 1-yr training period, O_2peak was increased by 20% ($P < 0.05$). However, neither O_2 at VT intensity nor workload at VT and peak at the end of the study were different from baseline values. O_2, workload, heart rate, and RER at VT and peak were similar between PL and CR at any time of the study. Furthermore, neither the training nor Cr intake per se significantly impacted blood pressure.

Muscle Force Measurements

Six-months follow-up. At baseline, maximal isometric knee extension force (Fmax) was 143 ± 7 N•m in PL vs. 140 ± 7 N•m in CR (not significant) (Fig. 1). In PL, the training intervention increased Fmax by 7% to 153 ± 8 N•m by 3 mo ($P < 0.05$); yet at 6 mo, values had reverted to baseline (146 ± 8 N•m). Fmax values in CR (3 mo: 146 ± 7 N•m; 6 mo 136 ± 8 N•m) were similar to PL at any time of the study. Dynamic force and fatigue were measured during an exercise test that consisted of two bouts of 30 maximal contractions with a 2-min rest pause in between. Because power curves were independent of either training or CR, Table 2 only shows mean dynamic torque outputs per bout. In PL, power output after 3 and 6 mo of training was not significantly different from baseline. Dynamic torque curves during either bout 1 or 2 were similar between PL and CR at any time of the study.

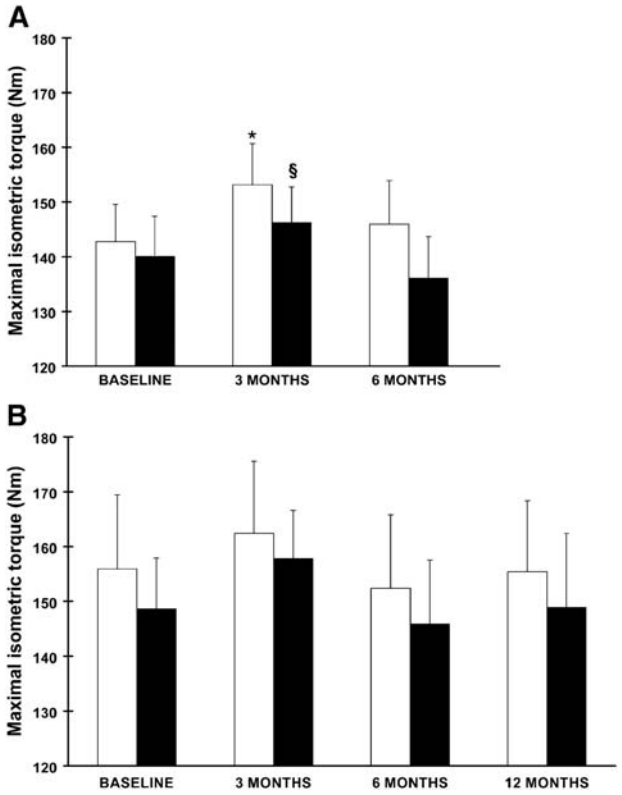

Fig. 1. Effect of creatine intake in conjunction with fitness training on static force of the knee extensor muscles in men 55–75 yr of age.

Values are means ± SE of 23 and 10 observations for the 6-mo (A) and 1-yr follow-up (B), respectively. Maximal isometric torque of the knee extensor muscles of the right leg was assessed using an isokinetic dynamometer before (baseline) and after 6 and 12 mo of fitness training combined with either placebo (open bars) or creatine intake (5 g/day; solid bars). *$P < 0.05$ and $P = 0.08$ compared with the corresponding baseline value. See METHODS for further details.

Table 2. Effect of creatine intake in conjunction with fitness training on dynamic force of the knee extensor muscles in men 55-75 yr old

	Baseline		6 mo		12 mo	
	Placebo	Creatine	Placebo	Creatine	Placebo	Creatine
6-mo follow-up (n = 23)						
Bout 1	92 ± 3	92 ± 4	98 ± 5	94 ± 5		
Bout 2	86 ± 3	98 ± 4	93 ± 4	90 ± 4		
1-yr follow-up (n = 10)						
Bout 1	94 ± 5	95 ± 7	96 ± 9	101 ± 7	98 ± 8	102 ± 8
Bout 2	95 ± 7	88 ± 7	91 ± 7	93 ± 7	93 ± 5	93 ± 7

Values are means ± SE and represent dynamic torque (in N·m) of 23 and 10 observations for the 6-mo and 1-yr follow-up, respectively. Dynamic torque was measured on an isokinetic dynamometer during 2 bouts of 30 maximal contractions of the knee extensor muscles, interspersed by a 2-min rest period. Mean torque was calculated for each bout before (baseline) and after 6 and 12 mo of fitness training combined with either placebo or creatine intake (5 g/day). See METHODS for further details.

One-year follow-up. Changes in F_{max} during the initial 6 mo of the follow-up mirrored the changes measured in the total group of subjects. However, because of the smaller number of observations, the increase (+4%) in Fmax after 3 mo of training was not statistically significant. Accordingly, Fmax at the end of the study was similar to baseline. Dynamic torque outputs during the dynamic fatigue test were not altered either by the training per se or by CR.

Body Composition

Six-months follow-up. Baseline values for body weight, percent body fat, and fat-free mass were similar between PL and CR (Table 3). Compared with baseline, body weight tended to decrease by 6 mo of training in PL ($P = 0.07$), whereas it was stable in CR. However, analysis of covariance did not yield a significant treatment effect for body weight. In PL, percent body fat slightly decreased by 6 mo of training (-1.1%, $P < 0.05$), whereas fat-free mass slightly increased ($P < 0.05$). Training-induced changes of both percent body fat and fat-free mass were similar between PL and CR.

Table 3. Effect of creatine intake in conjunction with fitness training on body composition in men 55-75 yr old

	Baseline		6 mo		12 mo	
	Placebo	Creatine	Placebo	Creatine	Placebo	Creatine
6-mo follow-up (n = 23)						
Body weight, kg	81.3 ± 2.5	79.2 ± 2.3	80.4 ± 2.5†	79.2 ± 2.3		
Body, fat, %	26.1 ± 1.0	25.9 ± 0.9	24.9 ± 1.0*	24.8 ± 0.8*		
Fat-free mass, kg	59.6 ± 1.2	58.4 ± 1.4	59.9 ± 1.0*	59.3 ± 1.4*		
1-yr follow-up (n = 10)						
Body weight, kg	81.9 ± 2.6	80.9 ± 4.3	80.3 ± 2.8	81.0 ± 4.3	80.3 ± 2.5	81.3 ± 4.3
Body fat, %	25.7 ± 0.9	27.2 ± 1.2	23.8 ± 1.1*	25.8 ± 1.2*	24.0 ± 1.0*	26.2 ± 1.2*
Fat-free mass, kg	60.8 ± 1.6	58.7 ± 2.6	61.0 ± 1.7	59.9 ± 2.7*	60.9 ± 1.6	59.8 ± 2.7*

Values are means ± SE of 23 and 10 observations for the 6-mo and 1-yr follow-up, respectively. Body composition was assessed by hydrostatic weighing before (baseline) and after 6 and 12 mo of fitness training combined with either placebo or creatine intake (5 g/day). *$P < 0.05$ and †$P = 0.07$ compared with the corresponding baseline value. See METHODS for further details.

One-year follow-up. Changes of body weight, percent body fat, and fat-free mass up to 6 mo follow-up paralleled changes observed for the total group of subjects. Compared with baseline, in PL at the end of the study, body weight and fat-free mass were not significantly changed. However, percent body fat was decreased by 1.7% ($P < 0.05$). There were no significant differences for the body composition measurements between PL and CR at any time of the study.

Muscle Biochemistry and Histochemistry

Six-months follow-up. Muscle Cr, PCr, and TCr content at baseline were similar between PL and CR, yet ATP content was slightly higher in CR ($P < 0.05$) (Table 4). Furthermore, in PL, values at month 6 were similar to baseline. In CR, compared with baseline, Cr (+21%, $P < 0.05$) and TCr (+5%, $P = 0.07$) increased, whereas PCr and ATP were stable. As shown in Fig. 2, a high negative correlation ($r = -0.78$, $P < 0.05$) was found between initial muscle TCr and the increase of muscle TCr produced by 6 mo of CR.

Table 4. Effect of creatine intake in conjunction with fitness training on muscle biochemistry in men 55-75 yr old

	Baseline		6 mo		12 mo	
	Placebo	Creatine	Placebo	Creatine	Placebo	Creatine
6-mo follow-up						
ATP	17.2 ± 0.7	19.7 ± 0.9*	18.0 ± 0.7	19.1 ± 1.3		
Creatine	45.2 ± 2.4	42.2 ± 1.9	46.5 ± 2.8	50.9 ± 2.5‡		
Phosphocreatine	98.1 ± 2.8	103.6 ± 5.7	93.0 ± 3.6	101.6 ± 3.6		
Total creatine	143.4 ± 3.1	145.8 ± 6.1	139.5 ± 4.2	152.5 ± 5.5†		
1-yr follow-up						
ATP	17.7 ± 1.1	19.7 ± 0.9	19.2 ± 1.0	17.5 ± 1.7	18.7 ± 1.0	18.5 ± 2.4
Creatine	43.0 ± 2.6	43.7 ± 2.8	42.4 ± 3.4	53.2 ± 4.8	42.0 ± 3.1	50.0 ± 4.3
Phosphocreatine	102.3 ± 4.2	91.4 ± 8.3	94.6 ± 7.0	103.6 ± 3.4	94.7 ± 3.8	114.2 ± 5.0‡
Total creatine	145.3 ± 5.4	135.1 ± 9.8	136.9 ± 7.5	156.8 ± 7.6	136.7 ± 4.4	164.1 ± 2.0‡

Values are means ± SE (mmol/kg dry wt) of 13 (placebo) and 12 (creatine) observations in the 6-mo follow-up vs. 7 (placebo) and 6 (creatine) observations in the 1-yr follow-up. Total creatine was calculated as the sum of creatine and phosphocreatine values. Muscle biopsies were taken from the m. vastus lateralis of the right leg before (baseline) and after 6 and 12 mo of fitness training combined with either low placebo or creatine intake (5 g/day). *$P < 0.05$, †$P = 0.07$ compared with the corresponding placebo value. ‡$P < 0.05$ compared with the corresponding baseline value. See METHODS for further details.

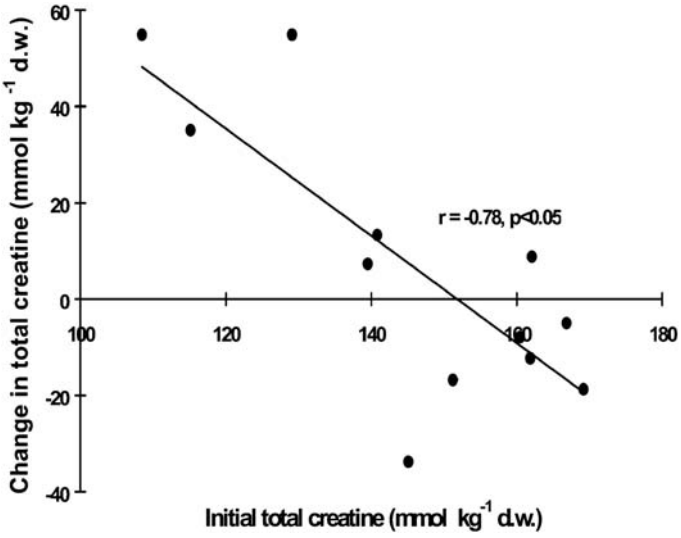

Fig. 2. Correlation between the individual changes in muscle total creatine content and initial muscle total creatine content after 6 mo of creatine supplementation in men 55–75 yr of age. Muscle biopsies (n = 12) were taken from the m. vastus lateralis of the right leg before and after 6 mo of fitness training combined with creatine intake (5 g/day). Total creatine was calculated as the sum of creatine and phosphocreatine values. dw, Dry weight. See METHODS for further details.

One-year follow-up. Muscle ATP, Cr, PCr, and TCr content at 0 and 6 mo were similar to the values found in the total group of subjects. However, because of the smaller number of observations, changes were not statistically significant. In PL, at the end of the 1-yr training intervention, ATP, Cr, PCr, and TCr values were similar to baseline. Conversely, in CR compared with baseline, PCr and TCr were 20–25% higher ($P < 0.05$), whereas Cr and ATP were unchanged. However, no statistically significant treatment effects for either PCr or TCr were detected. Because of the labor-intensive nature, histochemical analyses were only performed in subjects who completed the 1-yr follow-up and in whom muscle biopsies were available (PL: n = 7; CR: n = 6). As shown in Table 5, neither mean fiber area nor fiber area relative to total cross-sectional area for either type I, IIa, and IIx/b fibers significantly changed during the study in either group. Furthermore, in PL, relative type I, IIa, and IIx/b fiber number was stable throughout the study. Conversely, in CR compared with baseline, the relative distribution of type IIa fibers increased by 40%, whereas type IIx/b number decreased by 70% ($P < 0.05$). However, the latter changes were not significantly different from PL.

Table 5. Effect of creatine intake in conjunction with fitness training on muscle histochemistry in men 55-75 yr old

	Baseline		6 mo		12 mo	
	Placebo	Creatine	Placebo	Creatine	Placebo	Creatine
Mean fiber area, μm^2						
Type I	3,744 ± 334	4,509 ± 628	4,123 ± 311	4,551 ± 190	4,163 ± 335	4,585 ± 261
Type IIa	3,630 ± 234	4,016 ± 336	4,107 ± 542	4,422 ± 404	4,103 ± 199	4,657 ± 478
Type IIx/b	3,069 ± 241	3,340 ± 201	3,832 ± 477	4,011 ± 429	3,480 ± 193	4,251 ± 477
Relative area, %						
Type I	44.0 ± 6.9	55.3 ± 5.4	46.4 ± 6.4	57.2 ± 8.8	45.8 ± 2.7	55.6 ± 6.1
Type IIa	38.4 ± 7.0	29.0 ± 7.1	38.7 ± 6.2	32.5 ± 7.5	40.1 ± 3.5	40.3 ± 5.4*
Type IIx/b	17.6 ± 3.4	15.0 ± 3.3	14.6 ± 3.3	9.7 ± 6.5	14.0 ± 3.2	5.5 ± 2.2*
Relative number, %						
Type I	41.8 ± 5.9	52.2 ± 5.5	45.1 ± 5.0	55.3 ± 8.7	44.5 ± 2.1	55.2 ± 5.2
Type IIa	37.1 ± 5.8	29.2 ± 6.6	39.2 ± 5.6	32.5 ± 7.3	39.4 ± 4.0	40.3 ± 4.5*
Type IIx/b	20.6 ± 3.8	17.3 ± 4.7	15.5 ± 3.1	11.4 ± 3.7	16.1 ± 3.6	6.4 ± 2.7*

Values are means ± SE of 7 (placebo) and 6 (creatine) observations. Muscle biopsies were taken from m. vastus lateralis of the right leg before (baseline) and after 6 and 12 mo of fitness training combined with either placebo or creatine intake (5 g/day). *$P < 0.05$ compared with the corresponding baseline value. See METHODS for further details.

Clinical Chemistry (Data Not Shown)

To investigate the safety profile of long-term Cr intake, routine blood and urine clinical chemistry screening tests were performed during the 6-mo and 1-yr follow-up. All values remained within the normal clinical range throughout the study, with no significant differences between PL and CR. In CR, urinary Cr excretion obviously increased from 0.76 ± 0.12 g/24 h at baseline to 3.46 ± 0.26 g/24 h throughout the 1-yr period of CR. During the course of the 1-yr intervention period, plasma Cr concentration also slightly increased from 1.13 ± 0.02 mg/dl at baseline to 1.24 ± 0.04 mg/dl. Blood lipids were only measured in the 1-yr follow-up group. There were not differences either within or between the groups for total cholesterol, HDL-cholesterol, and LDL-cholesterol at any time of the study. However, at 12 mo of training, compared with PL (141 ± 18 mg/dl), triglycerides were lower ($P < 0.05$) in CR (88 ± 14 mg/dl).

Discussion

In this study, we investigated the potential of oral CR to improve physical fitness (8) in older men. Increasing the level of physical activity is a first-line strategy to improve fitness in older people (8, 29, 44). It is known from studies in young volunteers that CR can enhance the beneficial effects of resistance training on muscular functional capacity (48, 50). Therefore, we used CR as a "therapeutic" intervention additive to an exercise training program. Our data show that long-term oral Cr intake at a rate that has been proven to be effective in young subjects, does not enhance physical

fitness in older men.

Functional capacity of skeletal musculature is an important component of physical fitness (8). Deterioration of muscle performance capacity due to atrophy can reduce mobility and thus impair the quality of life in older individuals. On the basis of recent observations in young healthy volunteers (27, 34, 48, 50), it was reasonable to assume that CR might stimulate the effects of resistance exercise training on muscle force and power output in the elderly (46). We assessed static and dynamic strength of the knee extensor muscles on an isokinetic dynamometer. The training program, which among other exercises involved weight-lifting exercises for the knee extensors (leg press and leg extension), slightly increased Fmax most prominently during the initial stage of the training intervention. Hereafter, Fmax surprisingly normalized, despite regular training attendance (2.5 training sessions/wk) and normal progress of the training intensity. At present, it is unclear why by 6 mo of training the adaptations that had been elicited with 3 mo of training had been lost. Furthermore, Cr intake did not beneficially impact muscle force and power production, either in the total group of subjects (6-mo follow-up) or in the subgroup of subjects completing the full 1-yr training intervention (see Fig. 1). There is evidence from one study (50) to indicate that CR may enhance the effects of a resistance training program on muscle strength by facilitating the progress of training workloads. However, analysis of the training diaries showed that workloads of the weight-lifting exercises were similar between the placebo and CR subjects at any time of the study. Consistent with our present findings, one earlier study, performed by Bermon et al. (7), found no benefit to combining 8 wk of heavy resistance training with Cr intake in older persons. Conversely, two other studies reported improved muscle strength characteristics after either 12 (11) or 14 wk (10) of Cr intake in conjunction with a heavy resistance training program in men >60 yr old (11).

Another factor contributing to physical fitness as well as health is body composition (8). Increased fat-free mass relative to fat mass, by virtue of the high potential of energy turnover in skeletal muscle tissue, can significantly contribute to improved health status. There is evidence from long-term CR studies to indicate that supplementary oral Cr intake in conjunction with resistance training can stimulate muscle hypertrophy (27, 34, 47, 48, 50). Fat-free mass, assessed by hydrostatic weighing, was only marginally increased by the resistance training program used, and there was no additional benefit from the ingestion of Cr (Table 3). Accordingly, fat mass and body weight were not affected by CR. Thus the older subjects enrolled in this study clearly did not exhibit the increase of body weight inherent to CR in samples of young subjects (26, 30, 47). In fact, the failure of CR to increase fat-free mass probably also largely explains the unchanged F_{max}.

Functional capacity of the cardiorespiratory system also is an important

factor contributing to physical fitness. We evaluated cardiorespiratory fitness by means of a maximal exercise test on a bicycle ergometer. In fact, there is no clear rationale from literature data to anticipate that CR might enhance endurance exercise capacity (26, 30, 47). However, because the ability to cope with high workloads in cycling in older individuals is often limited by peripheral muscle weakness rather than by cardiorespiratory factors (2), we assumed that CR in this population might enhance cycle performance in both training and testing. However, training workloads were identical between groups from the start to the end of the study. Furthermore, indexes of submaximal and maximal endurance exercise performance during the maximal exercise test were significantly improved by the training intervention, yet were independent of CR.

Our present findings clearly indicate that the potential of CR to enhance muscular functional capacity, which is explicit in samples of young healthy volunteers (26, 30, 47, 48), is absent in the population of men between 55 and 75 yr of age studied here. Two obvious arguments can be cited to explain the apparently differential response of younger and older individuals to CR. First, it is well established that low initial muscle Cr content predisposes to good responsiveness to CR (24, 25). In the present study, we showed a similar relationship also to exist in older individuals: CR produced the largest increase of muscle TCr content in these subjects with the lowest initial values (see Fig. 2). However, because mean initial TCr contents were substantially higher [140–145 mmol/kg dry wt (dw)] than common values for young healthy subjects (115–125 mmol/kg dw) (24, 25), the increases of muscle Cr content due to supplementation on the average were very small. Similar findings were reported by Rawson et al. (41), who found muscle PCr content to be 20% higher and the response to acute CR to be smaller in old (70 ± 3 yr) compared with young (24 ± 1 yr) subjects. However, it is important to note that the duration of the present intervention trial (1 yr) was substantially longer than any earlier CR study in either young volunteers (27, 48) or older subjects (7, 10, 11, 42). In this respect, there are some data to suggest that the effects of short-term Cr intake may fade on long-term supplementation (13, 27, 48, 50). We thus cannot exclude that Cr ingestion produced some beneficial effects on muscle performance capacity during the initial stage of the intervention, which then disappeared by 6 mo of training. Two studies found 7 days (23) and 8 wk (11) of CR to cause a small increase of dynamic and isometric muscle force in elderly subjects. Accordingly, Brose et al. (10) recently demonstrated that, compared with placebo, CR in conjunction with 14 wk of heavy resistance training increased fat-free mass and isometric knee extention torque in male subjects by 3 and 25%, respectively. However, these investigators also reported that the gain in isometric ankle dorseflexion torque in female subjects after combined Cr intake and resistance training was smaller compared with their placebo counterparts.

Some (1, 17), but not all (49), studies have reported CR in combination

with resistance training to have a cholesterol-lowering effect. In this study in older individuals, we could not demonstrate a beneficial effect of supplementary Cr intake on either blood total cholesterol or on the HDL- and LDL-cholesterol fractions measured by routine clinical screening tests. Cholesterol levels were stable in either experimental group throughout the study, which also indicates that the exercise training-program per se did not alter blood lipid profile. This is probably explained by the fact that the training volume did not meet the threshold to decrease blood lipids (15, 16).

Long-term data with regard to potential adverse side effects of CR are very scarce. With the exception of two negative case reports in patients with preexisting renal disease (33, 40) and one study in Sprague-Dawley rats serving as a model for cystic renal disease (18), data to prove that CR per se could harm renal function in healthy individuals are entirely lacking (38, 39). In the present study, Cr intake at a rate of 5 g/day for 1 yr did not alter urinary albumin excretion. Furthermore, Cr ingestion produced a small, yet insignificant, increase of plasma Cr concentration in the early stage of the intervention period, but no further increase was observed from month 3 to month 12 of the study. However, four subjects of the Cr group vs. only one in the placebo group (2 test; not significant) developed ST depression during ECG stress testing during phase I of the intervention. Although the incidence of ST depression was not statistically significant between the experimental groups, this issue deserves particular attention in future studies, in particular in subjects at risk of or afflicted by cardiovascular disease.

It may be argued that CR did not enhance muscular functional capacity because the resistance training workloads used (20–30 RM) were too low. Indeed, it is clearly established that high workloads (5–10 RM) are needed for resistance training to produce muscle hypertrophy in older individuals (20, 21). Therefore, we cannot exclude that older persons involved in heavy resistance training, in contrast with the moderate resistance plus cardiorespiratory training program used in the present study, still might benefit from Cr intake. However, only a marginal fraction of the older population is involved in heavy resistance training. In fact, the training volume and intensity used in this study even largely exceed the level of physical activity typical to the vast majority of the sedentary Western older population, and there is no evidence from literature data that long-term CR alone, in the absence of exercise training, can beneficially impact neuromuscular performance capacity. In addition, the potential of exercise training programs to improve performance-related measures of functional capacity in older individuals as a rule is small (32), which is in keeping with our present observations.

In conclusion, the present study clearly shows that CR at a rate of 5 g/day is not an effective intervention to enhance physical fitness in men 55–75 yr of age enrolled in an exercise training program involving both endurance

exercise and moderate resistance training.

Disclosures

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Footnotes

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Effect of dietary creatine on skeletal muscle myosin heavy chain isoform expression in an animal model of uraemia

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Abstract

Background: Chronic renal failure is accompanied with muscle dysfunction and myopathy, characterized by muscle weakness and increased fatigue. Myosin heavy chain (MHC) is the principal structural protein that controls the intrinsic contractile properties of striated muscle. Creatine is widely used as an ergogenic nutritional supplement in sportsmen. This study investigates the effect of creatine supplementation on the myosin heavy chain expression in the setting of uraemic myopathy.

Methods: Male Wistar rats were either sham-operated or subtotal nephrectomized and received a control diet or creatine (2% w/w) supplemented diet. After 4 weeks of treatment, serum creatinine, creatine, urea and creatinine clearances were determined. MHC isoforms were determined electrophoretically in the extensor digitorum longus (EDL) and soleus muscle.

Results: Creatinine clearances were lower in nephrectomized animals, but similar in creatine supplemented and control diet animals. Nephrectomized animals had significantly higher MHC IIb and lower MHC IIx isoform expression in the EDL muscle, compared to sham-operated animals. In the soleus muscle MHC IIb expression was increased in nephrectomized animals. Creatine supplementation reversed the MHC transitions observed in uraemia in the soleus muscle, but not in the EDL muscle.

Conclusion: We observed altered expression of MHC isoforms in uraemia. Fast MHC IIb isoforms in uraemia were increased, whereas MHC I and IIx isoform predominate in control animals. Dietary creatine supplementation reversed the altered MHC expression during uraemia in slow-twitch, but not in fast-twitch muscle.

Key words:

Creatine supplementation, myosin heavy chain isoform, end stage renal disease

Abbreviations

ESRD, end stage renal disease ; MHC, Myosin Heavy Chain

Introduction

Chronic renal failure and especially dialysis is associated with muscle dysfunction [1]. The progressive loss of skeletal muscle mass leads to general weakness, low mobility and a general decrease in quality of life.
Apart from a specific uraemic myopathy, which is mainly caused by an abnormal vitamin D metabolism there are numerous other causes of muscle dysfunction in uraemia such as inactivity, malnutrition, neuropathy, underlying diseases, drugs and ischemia [2]. The progression of uraemic myopathy runs parallel with the decline in renal function. Significant correlation was described between glomerular filtration rate and muscle peak oxygen uptake [3]. Animal studies revealed several biochemical abnormalities, such as mitochondrial alterations, disturbed intracellular calcium transport, myofibrillar ATPase activity and abnormal nucleotide and phosphocreatine concentrations, contributing to disturbances in force generation and muscle wasting [2,3]. Proteolysis is accelerated in the uraemic muscle by activation of the ubiquitin-proteasome system through metabolic acidosis, low insulin sensitivity and glucocorticoids [4].
Muscle contraction is biochemically regulated by interaction between actin and myosin [5]. Myosin exhibits enzymatic properties catalyzing ATP hydrolysis during contraction. Myosin heavy chain (MHC) is the main determinant of the myofibrillar ATPase activity and contractile properties of the muscle fiber [6]. Up to nine MHC isoforms have been described in mammalian muscle, exhibiting different ATPase activity and accounting for the differences in contractile properties between fast and slow skeletal muscles. In adult rodent skeletal muscle four isoforms are generally described. MHC I is predominant in slow-twitch muscle and exhibits low ATPase activity, whereas MHC IIa, IIx and IIb are fast MHC isoforms with increasing ATPase activities. Slow-twitch muscle, with predominant expression of MHC I isoforms are more resistant to fatigue, compared to fast-twitch muscles with predominant type II fibers. The shortening velocity of the sarcomere however is lower in fibers with predominant MHC I, compared to fibers with predominant MHC II. Muscle fiber type is not static, but should be regarded as dynamic and capable of changing type in response to altered functional demand, innervation or metabolic conditions. The fiber type distribution of the muscles is a dynamic state constantly adjusting to the current conditions [5,7,8].
Transitions in MHC isoforms have been demonstrated in several animal models [7,8]. Models resulting in muscle atrophy such as hindlimb suspension or weightlessness induce phenotype transitions from slow to fast MHC isoforms, whereas models producing hypertrophy exhibit a shift in MHC towards slower isoforms. Fiber type transitions generally occur gradually and sequentially from slow to fast isoforms (I >IIa>IIx>IIb), or in the reverse order, though recent evidence suggests that shifts in MHC can proceed more dramatically and do not need to follow this strict

sequence [7,9-10] . In humans, marathon runners and ultra-endurance athletes exhibit a remarkably high percentage type I fibers in their major muscles, whereas sprinters and power lifters have predominantly IIa/IIx fibers. Apart from this training effect, MHC composition of the muscles is genetically determined [5,7-8].

Creatine [11-13] is increasingly popular as an ergogenic supplement in an athletic population. Normal dietary intake and endogenous synthesis in kidney and liver compensate for a daily loss as creatinine of about 2%. Creatine uptake in muscle is mediated by a specific sarcolemmal creatine transporter. Intramuscular creatine is phosphorylated to phosphocreatine by means of creatine kinase. Phosphocreatine serves as a temporal and spatial energy buffer in muscle, preventing a decrease in the ATP/ADP ratio during sudden increases in energy expenditure such as muscle contraction [11-12]. Furthermore the phosphocreatine/creatine kinase system acts as an energy carrier between sites of energy production (mitochondria) and sites of energy consumption (e.g. myofibrils). Short- and long-term creatine supplementation results in augmented intramuscular creatine concentrations and has been associated with increased muscle performance during high-intensity exercise in healthy volunteers. In addition to these reports in healthy subjects several studies have been performed studying the effect of creatine supplementation on muscle function in patients with neuromuscular or neurodegenerative diseases, or in animal models of these diseases [13-14].

The present study investigates the expression of MHC isoforms in an animal model of chronic uraemia. The effect of uraemia and creatine supplementation on MHC transitions in both slow (M. soleus) and fast muscles (M. extensor digitorum longus) were studied using animals with normal and impaired renal function.

Materials and Methods

Animal procedures

Male Wistar rats weighing 200-230 g were obtained from Iffa Credo (Brussels, Belgium). All animal care was in accordance with local prescriptions and the NIH Guide for the care and use of laboratory animals. Renal failure was induced by reducing renal mass using a standard procedure of subtotal nephrectomy as described before [15-16]. Rats were anesthetized with halothane (Fluothane, Astra-Zeneca) and a flank incision was made exposing the kidney. The upper and lower pole of the left kidney were cryoablated, followed 1 week later by a right nephrectomy. The sham procedure consisted of a flank incision without tissue destruction.

Animals were randomly allocated to 4 groups: 1. sham-operated, control diet (n=10); 2. sham-operated, creatine-supplemented diet (n=10); 3.

subtotal nephrectomized, control diet (n=10); 4. subtotal nephrectomized, creatine-supplemented diet (n=10). The animals were sedentary and provided free access to food and drinking water.

Creatine-supplementation was started 1 week after the last surgical procedure by addition of 2% creatine monohydrate (w/w; Sigma) to the diet. Control diet consisted of a soy-based maintenance chow (RM1, Special Diet Services, Witham, UK), containing 14 % protein.

Urine and blood samples were collected at the start and after 28 d of creatine-supplementation. Body weight and individual food intake were recorded on these occasions.

Animals were sacrificed by cervical dislocation under pentobarbital anesthesia (Nembutal, Sanofi) and hindlimb muscle were quickly excised, trimmed of connective tissue and frozen in liquid nitrogen until further processing.

Myosin heavy chain isoforms

Frozen muscles were homogenized by hand in 10 volumes of cold homogenization buffer [250 mmol/L sucrose, 100 mmol/L KCl, 5 mmol/L EDTA and 20 mmol/L Tris] in glass tissue grinders. The homogenates were further processed to prepare washed myofibrils [17]. The washed myofibrils were further diluted with sample buffer [62.5 mmol/L Tris (pH 6.8), 25 % glycerol, 1% sodium dodecyl sulphate, 2.5 % 2-mercaptoethanol and 0.01% bromophenol blue] and heated for 4 min at 95° C.

Myosin heavy chain isoforms were separated and analyzed by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) according to Talmadge and Roy [18]. Gels were run for 24h at 70 V (constant voltage) using 0.75 mm mini-slab gels (Mini-Protean II, BIORAD). The stacking gel consisted of 4% bis-acrylamide (1:50), 30% glycerol , 70 mmol/L Tris(hydroxymethyl)aminomethane (Tris) (pH 6.8), 4 mmol/L EDTA and 0.4% SDS. The separating gel was composed of 8 % bis-acrylamide (1:50), 30% glycerol, 0.2 M Tris (pH 8.8), 0.1 M glycine and 0.4% SDS. Upper running buffer consisted of 100 mM Tris, 150 mM glycine and 0.1% SDS. Lower running buffer consisted of 50 mM Tris, 75 mM glycine and 0.05% SDS. All reagents were of electrophoretic grade. Two µg of washed myofibrillar protein were loaded on the gel. Gels were stained with Coommasie Blue and scanned and the bands quantitated using Gel-Pro analyzer software. The different myosin isoforms were identified according to their migration. Myosin heavy chain isoforms were determined in both slow (M. soleus) and fast-twitch muscle (M. extensor digitorum longus). Both muscles are well characterized and were chosen for their opposite contractile characteristics. M. soleus is a model for a slow antigravity muscle, whereas M. extensor digitorum longus is a fast non-weight bearing muscle.

Biochemical determinations

Creatinine clearances. Serum and urinary creatinine concentrations were determined using a non-compensated rate-blanked Jaffé method (Roche Diagnostics, Mannheim, Germany) on a Modular P analyzer (Roche Diagnostics) according to the manufacturer's procedure. Creatinine clearances were calculated based upon 24 h urine collections. Serum urea concentrations were determined enzymatically (Roche Diagnostics).
Serum and urine creatine concentrations were determined enzymatically as described before [19]. Total intramuscular creatine concentrations (mmol/wet weight) were determined colorimetrically according to Berlet [20].

Statistics

Data are expressed as mean ± SD. Comparison between groups was performed using a one-way ANOVA, followed by post-hoc Student-Newman-Keuls (SNK) testing. Differences were considered significant at $p \leq 0.05$. Correlation between parameters was examined using Spearman rank correlation analysis.

Results

Biometrical and biochemical characteristics

Subtotal nephrectomized animals exhibited lower body mass and renal function indices compared to sham-operated animals. No significant differences in body weight or food intake were observed between creatine supplemented and control diet groups in either subtotal nephrectomized or sham-operated groups (Table 1). Creatinine clearance rates were similar in creatine supplemented and control diet animals. In the subtotal nephrectomized group serum creatinine was higher in creatine supplemented animals, compared to control diet animals, whereas no differences in serum creatinine concentrations were observed in the sham-operated group (Table 1). Serum creatine and urinary creatine excretion was significantly higher in creatine supplemented animals. Total intramuscular creatine concentrations were similar in nephrectomized and sham-operated animals, but higher concentrations were observed in creatine supplemented animals than in control diet animals [35.9 ± 2.9 mmol/kg vs. 32.9 ± 3.0 mmol/kg ; $p=0.003$ (pooled data)].

Muscle mass

Muscle mass (Table 1) was signicantly lower in nephrectomized animals in both fast [M. extensor digitorum longus 171 ± 17 vs. 183 ± 15 mg; $p=0.024$ (pooled data)] and slow-twitch muscles [M. soleus 141 ± 19 vs. 158 ± 17 mg ; $p=0.005$) (pooled data)] . Higher soleus mass was observed in creatine supplementated nephrectomized animals compared to control diet nephrectomized animals ($p \leq 0.05$ (SNK)).

Table 1. Comparative biometrical and biochemical parameters in animals fed a control or creatine-supplemented diet with or without preexisting renal failure

Parameters	Sham operation		Subtotal nephrectomy	
	control diet (n = 10)	creatine diet (n = 10)	control diet (n = 10)	creatine diet (n = 10)
Body weight, g[a]	426 ± 18	416 ± 23	389 ± 23[b]	386 ± 24[b]
Food intake, g/day	17.2 ± 1.3	17.7 ± 3.4	18.5 ± 3.0	16.9 ± 4.3
Musculus soleus, mg[a]	155 ± 11	162 ± 21	133 ± 17[b]	149 ± 18[c]
Musculus extensor digitorum longus, mg[a]	188 ± 12	179 ± 16	166 ± 18[b]	176 ± 16
Serum creatinine, μM[a]	45 ± 6	49 ± 4	61 ± 7[b]	68 ± 10[b,c]
Serum urea, mM[a]	5.0 ± 1.0	5.3 ± 0.5	9.7 ± 2.2[b]	9.5 ± 1.5[b]
Creatinine clearance, ml/min[a]	1.6 ± 0.4	1.5 ± 0.2	1.1 ± 0.2[b]	1.1 ± 0.2[b]
Serum creatine, μM[a]	137 ± 69	702 ± 348[c]	108 ± 46	1,021 ± 435[b,c]
Urinary creatine, mM[a]	0.10 ± 0.05	117.4 ± 65.7[c]	0.05 ± 0.02	83.9 ± 20.1[c]
Total muscular creatine, mmol/kg[a]	32.6 ± 3.9	35.9 ± 3.5	33.2 ± 2.0	35.8 ± 2.4[c]

[a] $p \leq 0.05$, ANOVA multiple group comparison, [b] $p \leq 0.05$, subtotally nephrectomized group vs. respective sham-operated control group (post hoc SNK), [c] $p \leq 0.05$, creatine diet group vs. respective control diet group (post hoc SNK). Conversion to conventional units: creatinine: $\mu M \times 0.0113$ = mg/dl ; creatine: $\mu M \times 0.0131$ = mg/dl; urea: m$M \times 6.006$ = mg/dl.

Myosin heavy chain isoform distribution

Separation of all four adult skeletal MHC isoforms was attained using the protocol. A typical electropherogram is illustrated in Figure 1. Subtotal nephrectomized animals had a significantly different MHC isoform distribution, compared to sham-operated animals (Fig 2). An increased proportion of the fast-twitch isoform IIb was observed in control diet nephrectomized animals, compared to control diet sham-operated animals in both slow-twitch (M. soleus: 18 ± 5 % vs. 2 ± 2 %; $p \leq 0.05$ (SNK)) and fast-twitch muscle (M. extensor digitorum longus: 60 ± 5 % vs. 44 ± 6 %; $p \leq 0.05$ (SNK)). The MHC IIx expression in M. extensor digitorum longus (38 ± 5 % vs. 56 ± 4 %; $p \leq 0.05$ (SNK)) was significantly lower in nephrectomized control diet animals, compared to sham-operated control diet animals.

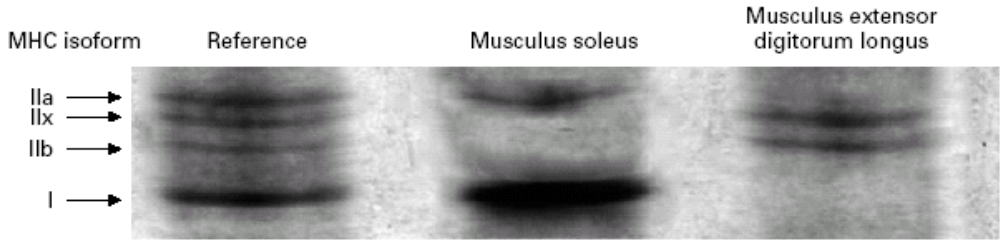

Fig. 1. Representative electrophoretic separation of the MHC isoforms. The reference lane (control) presents all four MHC isoforms, musculus soleus predominantly type I (slow) and musculus extensor digitorum longus predominantly fast MHC isoforms (MHC IIx and IIb).

Creatine supplementation was associated with lower MHC IIb expression ($p \leq 0.05$ (SNK) in the soleus muscle in the subtotal nephrectomized group. No difference in MHC isoform expression was observed between sham-operated creatine supplemented animals and sham-operated control diet animals in M. soleus (Fig 2).
No effect of creatine supplementation was observed on MHC isoform expression in the M. extensor digitorum longus in both sham-operated and subtotal nephrectomized animals.

In fast-twitch muscles the expression of myosin isoforms correlated with renal function. MHC IIx expression correlated negatively with serum creatinine ($r=-0.51$; $p=0.001$) (Figure 3) and serum urea ($r=-0.50$; $p=0.01$), but not with muscle mass (p=NS). MHC IIb expression correlated positively with serum creatinine ($r=0.50$; $p=0.01$) (Figure 3) and serum urea ($r=0.48$; $p=0.02$).
In slow-twitch muscles the relation of MHC expression and renal function failed to reach significance (MHC I expression vs. serum urea: $r= -0.32$: $p=0.06$). No significant relation was observed in M. soleus between the expression of fast MHC isoforms and renal function indices. In slow-twitch muscle expression of myosin heavy chain isoforms correlated with muscle mass (MHC I ~ M. soleus mass: $r= 0.41$; $p= 0.02$). In sham-operated animals no correlation between serum or muscular creatine and MHC isoform expression was observed in both fast and slow-twitch muscles. In subtotal nephrectomized animals, serum creatine concentrations inversely correlated with MHC IIb isoform expression: $r=-0.55$; $p=0.02$).

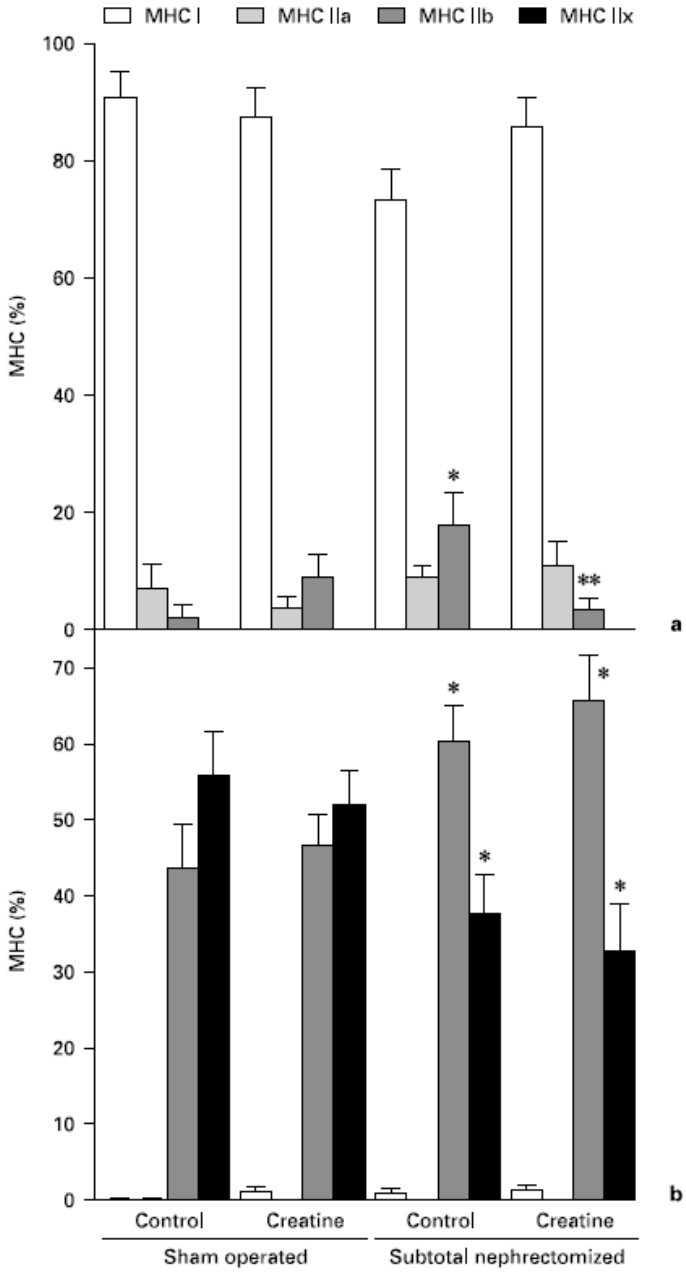

Fig. 2. Relative distribution of the MHC isoforms in sham-operated and subtotally nephrectomized animals in both extensor digitorum longus (b) and soleus muscles (a). * $p \leq 0.05$: subtotally nephrectomized animals compared to sham-operated control diet animals (SNK), ** $p \leq 0.05$: creatine-supplemented subtotally nephrectomized animals compared to control diet nephrectomized animals (SNK).

Discussion

The present study is the first to demonstrate altered MHC expression in animals with renal failure, compared to sham-operated animals. Ultra-fast IIb expression was increased in subtotal nephrectomized animals. The relative increase in expression of fast MHC isoforms has been described before in other animal models of muscle wasting.

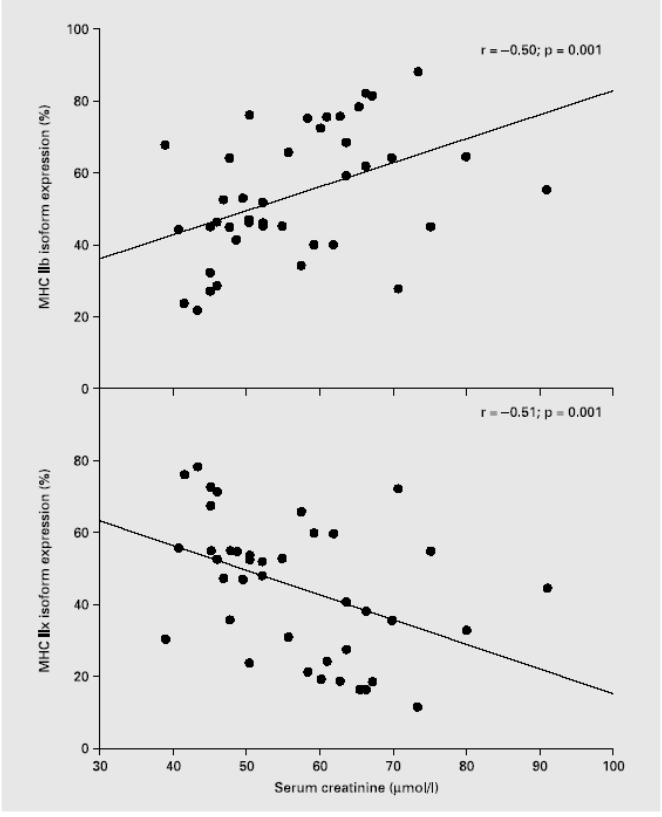

Fig. 3. Relationship between serum creatinine concentration and MHC isoform IIb (%) and IIx expression (%) in musculus extensor digitorum longus (Spearman's rank correlation analysis).

Microgravity during spaceflight and hindlimb suspension in animals induces muscle atrophy, together with significant changes in MHC isoform expression [7]. These adaptations are most prominent in slow muscle types and involve downregulation of the slow isoform MHC I and de novo expression of fast MHC IIx [21-22]. Cancer cachexia has been described to be associated with expression of fast MHC isoforms in soleus muscle.

Lower muscle mass, together with de novo expression of MHC IIb isoform expression at the expense of MHC I in the tumor bearing mice [23]. Congestive heart failure is also associated with lower exercise capacity and muscle wasting, as observed in uraemia. Skeletal MHC isoform expression is altered in rats with congestive heart failure with higher expression of MHC IIb at the expense of MHC IIx isoforms. These shifts in MHC isoforms are correlated to heart failure indices [24]. Histochemical analysis of skeletal muscle in uraemic subjects has mainly demonstrated a type II fiber atrophy. Type II fibers were smaller in uraemic subjects, compared to the reference population [1,25-26]. Light microscopy anomalies are mainly fiber atrophy, fiber splitting, increased intracellular lipid and moth-eaten fibers [25]. In this study we have examined MHC composition in animals using whole muscle homogenates and observed mainly a slow-to-fast transition in MHC composition. This observation is in line with other models of muscle wasting [21-24], though do not correspond with the above mentioned microscopic findings. However Clyne et al. described an inverse relationship between the proportion slow (type I) fibers and renal function [1]. This finding is in accordance with the observed slow-to-fast transition in our animal model. Comparison of animal and human studies are complicated by the presence of 3 fast MHC isoforms in rats, whereas humans exhibit only 2 fast MHC isoforms, by differences in methodologies and differences in muscle composition [7]. The soleus muscle in rats contains proportionally much more type I fibers, compared to human soleus which is more heterogeneous in fiber composition [7].
In humans resistance training and creatine supplementation demonstrated significant changes in MHC isoform composition [27-28]. Resistance training resulted in higher expression of slow MHC isoforms and a decreased expression of fast MHC isoforms. Combined training and creatine supplementation resulted in increased slow MHC I expression and decreased MHC IIx expression, compared to sedentary controls and trained placebo subjects. These data suggest that creatine supplementation can enhance training effects in humans [28].

Increased expression of fast MHC isoforms in both fast-twitch and slow-twitch muscles could affect contractile properties of the different muscle types in animals with renal failure. These transitions in MHC could account for the increased fatigability and dimished fatigue resistance observed in patients with renal failure. Fast MHC, especially IIb and IIx isoforms exhibit higher shortening velocities and ATP consumption rates but have higher fatigability [6,8]. The increase in fast MHC isoforms in our animal model of uraemia is mainly an increase in IIb isoforms (fastest), whereas disuse demonstrated mainly a decrease in type I and an increase IIa/IIx. Increases in MHC IIb isoform in M. soleus has been described during unloading, though the proportion was lower than reported here [21,29].

Diffee et al. reported comparable MHC IIb expression in M. soleus in tumor bearing mice as in our uraemic animals [23].
The MHC differences between sham-operated and nephrectomized animals could have arisen from changes in neuromuscular control [30]. Uraemia is associated with neuropathy [31] and could result in alterations in muscular innervation, apart from intrinsic muscular alterations. Neurons controlling fast- and slow muscle could differ in susceptibility to uraemic toxins and neuronal survival. These neuronal causes could partly influence MHC expression in skeletal muscle.
The observed MHC transitions in nephrectomized animals could also be attributed to decreased physical activity in these animals. Disuse has been associated with slow-to-fast transitions in MHC isoforms. However the observed MHC transitions by complete removal of mechanical load or nerve activity are less pronounced than the current changes observed in uraemia [5,7,22]. Moreover no training scheme was applied in this study protocol, therefore the differences in physical activity between the sham-operated and nephrectomized animals should be minimal.
The underlying mechanism causing MHC transitions in disease states, such as sepsis or cancer cachexia is poorly understood. Increased concentrations of circulating proinflammatory cytokines could influence muscle metabolism and could contribute to the altered muscle MHC isoform expression, observed in uraemia, cancer cachexia or heart failure [32]. Apart from acting on muscle energy metabolism, creatine could also interfere with the augmented inflammatory state as observed in uraemia. Creatine has been described to exhibit direct antioxidant properties. Creatine acted as a direct antioxidant against aqueous radical and reactive oxygen species [33-34]. These systemic effects of creatine supplementation could have influenced the proinflammatory state and circulating cytokines.

Serum creatinine concentrations were higher in creatine supplemented nephrectomized animals, compared to control diet nephrectomized animals, due to creatine-creatinine conversion. No effect of creatine supplementation on serum creatinine was observed in sham-operated animals. Previously we examined the effect of creatine supplementation on renal function and did not observe alterations in inulin or creatinine clearance nor in renal protein handeling [16]. No adverse effect of creatine supplementation on renal function could be demonstrated in this animal model of chronic uraemia [16].

Creatine supplementation increased expression of slow MHC isoforms of the soleus muscle in subtotal nephrectomized animals, therefore partially reversing the effect of uraemia on MHC expression. In fast-twitch muscle no effect of creatine supplementation was observed in either sham-operated or subtotal nephrectomized animals. These findings could have

therapeutic applications. Creatine supplementation could increase fatigue resistance and muscular performance in uraemia. Further research is warranted examinating the effects of creatine supplementation on muscular contractile properties and fatigue resistance in uraemic animals.

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Note added to manuscript in thesis:

The provided analgesia in relation to surgery in the animals is not mentioned in the manuscript. The analgesia was provided 48h post-operatively, by administration of buprenorphine IM (0.1 mg/kg; Temgesic, Schering-Plough).

In this study, creatine concentrations were determined in the white gastrocnemius muscle for technical reasons. In order to draw final conclusions, creatine concentrations should also be determined in the M. soleus and M. extensor digitorum longus separately. This allows differentiation between the effects of creatine on slow- and fast muscle types. Furthermore the effects of creatine on contractile characteristics should be studied in addition to the altered MHC-distribution.

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CHAPTER 4

General discussion & *future perspectives*

When the people of the world all know beauty as beauty,
there arises the recognition of ugliness.
When they all know the good as good,
there arises the recognition of bad.
Therefore being and non-being produce each other;
difficult and easy complete each other;
long and short contrast each other;
high and low distinguish each other;
sound and voice harmonize with each other;
beginning and end follow each other.

Tao Te King 2/81
Lao Tse

GENERAL DISCUSSION & FUTURE PERSPECTIVES

1. Major findings

This thesis addresses the effect of creatine supplementation on renal function, skeletal muscle metabolism and homocysteine metabolism in both laboratory animals and humans.
We considered creatine as an interesting molecule with possible application in renal failure patients. Two aspects of creatine metabolism are especially important in uraemia.
First, creatine is metabolically linked to the homocysteine metabolism. We hypothesized that creatine could lower the increased homocysteine concentrations associated with renal failure. In general, new homocysteine lowering therapies could be important in lowering cardiovascular risk in these patients.
Secondly, creatine is an ergogenic molecule in young athletes. Older subjects and especially uraemic patients are characterized by low muscle mass and low exercise tolerance. Creatine could increase the muscular performance and quality of life of these patients.
However, safety of prolonged creatine intake in conditions with diminished renal function was to be investigated, as only limited data are available on the prolonged intake of creatine. In this regard we have started by investigating the effects of creatine on renal function and uptake of creatine in our animal model as a prerequisite for further investigations (Taes (1) et al., 2003).
In our experiments with laboratory animals and in our studies in patients we did not observe any negative effect of creatine on the renal function. Creatine supplementation increased serum creatinine concentrations due to the elevated creatine-creatinine load without affecting glomerular filtration. Analytical interference could have influenced the apparent creatinine concentrations, although only to a minor extent (Taes et al., 2005).
In animals, creatine was found to decrease the plasma homocysteine concentration, whereas in dialysis patients no effect of creatine on plasma homocysteine concentrations was observed (Taes (2) et al., 2003; Taes (1) et al., 2004).
In laboratory animals we have investigated the myosin heavy chain expression in skeletal muscle and observed a protective effect of creatine in preventing the slow-to-fast isoform transition in myosin heavy chain during uraemic muscle wasting (Taes (2) et al., 2004). In this regard, treatment with creatine could be theoretically interesting in patients to prevent skeletal muscle wasting and increased fatigability. In aging men,

however, no major effect of creatine supplementation on exercise performance was observed in our study (Eijnde et al., 2003)

2. Analytical considerations

When evaluating kidney function in both rats and humans, it is important to use gold-standard methods such as inulin clearance or ^{51}Cr-EDTA-clearance rates (Kühnle et al., 1993). Calculated creatinine clearance rates give a good estimation of the renal function in conditions where the creatinine production is constant. When this condition is not met, creatinine clearance rates can provide a false estimation of the glomerular filtration rate. Tubular secretion of creatinine can also influence the calculated clearance rates. Creatine ingestion changes the steady state between creatinine formation and creatinine excretion, until a new equilibrium is set at higher (or lower) plasma creatinine concentration. In this situation, creatinine concentrations and creatinine clearance are no valuable markers of glomerular filtration. Independent markers of glomerular filtration are better suited in these conditions. The use of creatinine as a marker for glomerular filtration in several reports about creatine could have influenced the reported values for creatinine clearance and could explain the discrepancies in the literature about the effects of creatine on renal function (Van Liew et al., 1993). Moreover, as described in the introduction, the different creatinine assays suffer from interference with creatine (Taes et al., 2005; Weber et al. 1991). In the majority of the assays higher creatinine concentrations are reported with creatine ingestion and these interferences could have influenced the results.

3. Creatine and renal function

Models of renal mass reduction have been extensively used to investigate the pathogenetic mechanisms responsible for the progressive nature of chronic renal disease in humans. The 5/6 nephrectomy model with a partial reduction of the renal mass of one kidney and a collateral nephrectomy is commonly used (Gagnon et al., 1983).
A possible limitation of this animal model to study human renal injury is the time-frame in which the renal lesions occur. In this animal model the renal mass reduction is instantaneous and mimics poorly the situation in humans, where renal lesions occur gradually and progressive. Moreover, intrinsic renal disease is absent in these animals and does not mimic the course of renal disease in humans where the intrinsic renal disease contributes to the reduction in glomerular filtration. In general, the remnant kidney model is a good and widely used model for studying renal failure in various animal species. However the natural history of renal disease in these animals could be different from humans and could differ between the different animal models of renal disease.

In contrast to the absence of adverse effects of creatine on renal function in our animal model, other investigators have observed negative effects in animals. Edmunds et al (Edmunds et al., 2000) have investigated the effects of creatine on renal function in Han: Sprague-Dawley (SPRD)-cy rats. The Han:SPRD-cy rat is an animal model of inherited renal cystic disease that resembles human autosomal dominant polycystic kidney disease. Creatine supplementation resulted in greater cyst growth and worsened renal function in the Han: SPRD-cy rat, evidenced by greater kidney weights, renal cysts, serum urea concentrations and lower creatinine clearances. These authors conclude that creatine supplements could exacerbate disease progression in an animal model of cystic renal disease. Unfortunately no independent marker for glomerular filtration or renal protein handling was measured in this study and some methodological and physiological pitfalls were not taken into account. As mentioned before, creatine can interfere with the creatinine determinations and this interference could have influenced the results obtained for glomerular filtration. The difference in animal strain could also account for the differences between the study by Edmunds and our study as Esposito et al. demonstrated that the glomerular response to nephron reduction is dependent on the inbred strain of studied mice (Esposito et al., 1999). The use of animal experiments in evaluating biochemical effects of creatine can provide valuable information (Kreider, 2003), difficult to obtain in humans.

4. Creatine and homocysteine metabolism

In our studies, creatine decreased the homocysteine concentrations in rats, but not in humans. Several hypotheses can be advanced to explain this discrepancy.

First, in rats a higher dose (/kg body weight) of creatine was used than in humans. Theoretically, higher dosages in humans could have been efficient in lowering homocysteine concentrations. However, the attained plasma concentration of creatine was comparable between the laboratory animals and patients, owing to the lower residual renal function in the dialysis patients than in uraemic rats. Therefore, higher doses in humans would probably not have increased the efficiency of creatine in lowering homocysteine concentrations. In addition, patient compliance might have decreased as these patients were already taking several medications and gastrointestinal side effects were not uncommon. Finally, the plasma concentrations of guanidinoacetic acid were significantly diminished in the creatine supplemented dialysis patients, indicative for suppression of the endogenous creatine synthesis (Y. Taes, unpublished data).

Secondly, species-related differences could perhaps explain the difference in effect between humans and rodents. Considering homocysteine metabolism by the kidney, results obtained in the rat model showed decreased renal metabolism in uraemia. In rats about 75 % of plasma tHcy was found to be free, whereas in humans about 65-75 % of tHcy is bound to proteins by a disulfide bond. Only a small fraction of homocysteine circulates in a free form in human plasma, and is therefore available for glomerular filtration (Friedman et al., 2001; House et al., 1998). In dialysis patients protein binding of Hcy was even found to be higher in comparison to non-dialyzed chronic uraemic patients (Suliman et al., 1997). These differences in protein binding could be reflected in differences found in renal extraction of Hcy in humans and rats. In rats significant clearance of Hcy by the kidneys was observed (House et al., 1998), whereas in humans no arteriovenous difference in Hcy concentrations was observed. Van Guldener et al. measured homocysteine levels in the renal vein and renal artery of 20 patients with normal renal function undergoing a coronarography, in the fasting state, and they reported no significant difference (Van Guldener et al., 1998). Differences in renal handling of Hcy and in general Hcy-metabolism could account for the discrepancy in our studies. Another species-related aspect could be a possible difference in regulation of the creatine metabolism. The compartmentalization between the two key enzymes (AGAT, GAMT) varies from species to species (Wyss et al., 2000) and regulation and quantitative aspects can differ substantially. Another difference between rats and man is the nutritional intake. In particular, methionine intake plays a role in determining the fate of homocysteine, being remethylated to methionine or processed in the transsulphuration pathway (Selhub, 1999; Park et al., 1999).

5. Effects of creatine on skeletal muscle

Creatine has been extensively investigated in relation to skeletal muscle function in diverse exercise disciplines (Lemon, 2002; Rawson et al., 2003; Racette, 2003; Kreider, 2003; Paddon-Jones et al., 2004).

The use of creatine in therapeutic conditions in patients with neuromuscular disorders will be briefly addressed here in relation to the presented manuscripts.

Creatine has been studied in several neuromuscular disorders (Derave et al., 2003; Wyss et al., 2002). Three categories can be discriminated: (1) diseases related to creatine metabolism; (2) diseases with a disturbed creatine metabolism as a secondary symptom and (3) diseases without altered creatine metabolism.

In diseases of the first category such as GAMT- or AGAT-deficiency, creatine concentrations are low in various tissues. Creatine supplementation can result in normalization of the creatine stores and

clinical improvement. A defect in the creatine transporter can also be classed in this group, though the effects of creatine supplements in this condition are only minimal due to the absence of an active uptake mechanism. (Stöckler et al., 1994; Bianchi et al., 2000; Salomons et al., 2001; Cecil et al., 2001)
Gyrate atrophy due to a deficiency of the ornithine-aminotransferase, with inhibition of the creatine biosynthesis, together with mitochondrial cytopathies are classed in category 2. Creatine can improve muscular dysfunction of these patients, but not the loss of vision, associated with the disease (Heinanen et al., 1999; Sipilä et al., 1980; Sipilä et al., 1981;).
The third category groups several neuromuscular disorders, where creatine metabolism is not primarily affected. Creatine is supplemented to increase the survival of neurons or muscle cells or to improve neuronal or muscular function, as creatine can facilitate energy transfer from the mitochondria. Contradictory and disappointing results have been obtained in this group of patients.
In G93A mice, an animal model for amyotrophic lateral sclerosis, increased survival of these mice and improved neuromuscular control after creatine supplementation were described (Klivenyi et al., 1999; Zhang et al., 2003). Using the same transgenic animal strain (G93A), other investigators could not demonstrate any effect of creatine on muscular performance, muscle contraction characteristics or metabolites (Derave et al., 2003). In humans, no benefit of creatine was demonstrated in amyotrophic lateral sclerosis, considering muscular function (Ellis et al., 2004; Shefner et al., 2004; Groeneveld et al., 2003; Drory VE et al., 2002; Mazzini et al., 2001).
In Duchenne's muscular dystrophy, in vitro evidence suggested improved myotube formation and survival (Pulido et al., 1998). In humans with Duchenne's disease, creatine was found to increase the dominant hand isometric grip strength and lower excretion of N-terminal collagen telopeptides, indicative for lower bone resorption (Tarnopolsky et al., 2004).
In Kearns-Sayre (chronic progressive external ophtalmoplegia), a mitochondrial cytopathy, creatine supplementation did not improve skeletal muscle oxidative capacity (Kornblum, 2005).
In 81 patients with various neuromuscular diseases (mitochondrial cytopathies, neuropathic disorders, dystrophies-congenital myopathies and inflammatory myopathies), creatine supplementation increased body weight, handgrip, dorsiflexion, and knee extensor strength (Tarnopolsky et al., 1999).
In vitro studies identified creatine as an important neuroprotective factor for developing nigral dopaminergic neurons (Andres et al., 2005)
In animals, creatine was found to attenuate the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)- neurotoxicity, a model for Huntington's disease. Creatine protected against MPTP-induced loss of Nissl and tyrosine

hydroxylase immunostained neurons in the substantia nigra, indicative for attenuated dopamine depletion (Matthews et al., 1999). In the R6/2 mice, a transgenic animal model of Huntington's disease, creatine increased survival, motor performance and decreased neuronal atrophy and huntingtin aggregates (Dedeoglu et al., 2003; Andreassen et al., 2001). In humans with Huntington's disease, creatine did not improve functional, neuromuscular, and cognitive status. Creatine was supplemented at a dose of 5 g/d, sufficient to improve muscle function in healthy subjects (Verbessem et al., 2003).

In aging, we assumed that creatine could enhance muscular performance comparable to the ergogenic effects of creatine in young subjects. Short-term supplementation in older men slightly increased the isokinetic knee extension performance (Rawson (1) et al., 1999), whereas creatine supplementation of longer duration (1-2 months) did not improve muscular function (Rawson (2) et al., 1999; Bermon et al., 1998). Crusch (Crusch et al., 2001) demonstrated enhanced muscle strength, endurance and lean mass in addition to resistance training. The same authors (Candow et al., 2004) however could not demonstrate an effect of creatine withdrawal during training on strength, endurance and changes in lean mass. Brose (Brose et al., 2003) demonstrated increased isometric knee extension strength, increased fat-free and total body mass in creatine supplemented subjects, compared to placebo treatment, in conjunction with strength exercise training. Gotshalk (Gotshalk et al., 2002) showed positive effects of creatine on isometric, dynamic and functional muscle function tests in a population of older men. In our study we could not demonstrate any effect of creatine on muscular performance in these elder men. As in other studies the increase in muscular creatine concentrations was dependent on the initial creatine concentration. A low initial concentration predisposes to a good responsiveness to creatine. In our study with older men, high muscular creatine concentrations were found, in contrast to younger subjects, possibly limiting the responsiveness to creatine treatment. The duration of creatine supplementation is also important in considering these results. The effects of creatine, observed in short-term studies, may wear off in studies with longer duration. In senescence-accelerated mice (SAMP8), creatine supplementation was investigated in relation to contractile characteristics, creatine content and muscle morphology. In both soleus and extensor digitorum longus muscle, reduced phosphocreatine concentrations were observed with advancing age, without effect of creatine supplementation. In this animal model, creatine treatment did not influence the loss of muscle mass and contractility (Derave et al, 2005). An interesting aspect of creatine supplementation in elderly could be improvement of brain function. Creatine has been described to enhance brain performance in young vegetarians (Rae et al., 2003). Preserving brain function during aging is important in maintaining

quality of life. Creatine was described to attenuate ischemic brain infarction in a mice-model (Zhu et al., 2004). This neuroprotective property needs futher research, but provides an interesting approach to keep fit at an older age. Reviewing the muscular creatine concentrations in relation to the duration, there is a marked decrease in muscular creatine concentrations after 12 weeks of supplementation, suggestive for habituation (Derave et al., 2003). The origin of this habituation process remains unknown, although downregulation of the creatine transporter is possible. (Guerrero-Ontiveros et al., 1998). Beneficial effects of creatine on muscular function in patients with neuromuscular disorders were also only observed in short-term studies, whereas long-term studies failed to demonstrate major effects of creatine in this group of diseases (Derave et al., 2003).

In uraemia, specific alterations in skeletal muscle morphology have been described (Clyne et al., 1993; Campistol, 2002). The creatine metabolism could have been altered by the renal failure and the accumulation of uraemic retention products. Therefore we hypothesised that creatine supplementation could influence muscle metabolism in uraemia. Promising findings were observed in our animal model of chronic uraemia considering the protective effect of creatine on MHC-expression in the skeletal muscle (Taes (2) et al., 2004). Extrapolation of our animal data to patients with renal failure creates the impression that creatine could be helpful in treating the muscular dysfunction, associated with uraemia. Similarly, in untrained young subjects, a higher proportion of MHC I was observed with creatine supplementation, compared to sedentary and trained control subjects (Willoughby et al., 2001). However, creatine treatment in older men together with exercise training did not influence the muscular performance of these subjects.

Several remarks can be formulated on these opposing findings. Rats and humans differ in muscular composition. Especially, the M. soleus in the rat contains relatively more type I fibers, compared to humans (Pellegrino et al., 2003). This difference in muscular composition suggests that animal data could not be fully transposable to the human situation, also due to differences in contraction characteristics of single muscle fibers (Pellegrino et al., 2003). In this regard, biochemical effects of creatine on human MHC-expression could be smaller in aging men and without a measurable effect on contraction characteristics or muscular performance. Moreover creatine uptake can vary among species. In humans, intramuscular creatine concentrations increase more consistently compared to rodents or other animals. In horses, creatine uptake in muscle even seems absent after creatine loading (Schuback et al., 2000). In both rats, mice and guinea pigs, creatine supplementation was found to increase intramuscular creatine concentrations by about 30% (Ipsiroglu et al., 2001)

The effects of creatine on muscle function in dialysis have not been examined in large studies. Only Chang (Chang et al., 2002) reported in a small study, a decreased frequency of dialysis-associated muscle cramps, but muscular performance was not investigated. The absence of any beneficial effect of creatine on muscular performance in the aging men suggests that creatine could not prove useful in dialysis patients for increasing muscular performance. The muscular dysfunction in these patients is usually debilitating, with limited exercise tolerance and high fatigability. This finding, together with a multifactorial aetiology do not suggest a pronounced effect of creatine in this patient population. Also muscular creatine concentrations are not decreased in uraemia, limiting the responsiveness to creatine. Furthermore, these patients would only benefit from long-term creatine supplementation and with limited evidence available for long-term effect of creatine supplementation, this is less promising.

6. Future perspectives

Based on our data, the effect of creatine on muscular performance in aging men and by extrapolation in renal failure patients seems limited. As several publications with negative results on creatine and muscular function in humans with neuromuscular disorders have been published, it is to be expected that creatine will have only limited beneficial effect on muscular function in renal disease patients.
In this regard, a more appealing substance to study in relation to muscular function in renal disease patients is vitamin D (Visser et al., 2003; Wanic-Kossowska et al., 1996). Skeletal muscle has been identified as one of the non-classical target organs for vitamin D, by the presence of the vitamin D receptor (Bischoff-Ferrari et al., 2004). Treating elderly with vitamin D supplements reduced the number of falls and increased muscle strength (Kenny et al., 2003; Dhesi et al., 2004). The effects of vitamin D on skeletal muscle metabolism are still under investigation. The effect of vitamin D on muscle composition and contraction characteristics can prove interesting. Promoting physical exercise in renal failure patients could also help in limiting sarcopenia and increasing muscle strength.

An intriguing aspect of creatine metabolism is the effect of increased creatine ingestion on guanidino compound concentrations in renal failure. Guanidino compounds are structurally and metabolically related to arginine and creatine and have been described as uraemic toxins. Creatine could influence the concentration of the guanidino compounds in uraemia by the arginine-sparing effect of creatine.

Creatine could also play a role in Advanced Glycation End-product (AGE) formation, due to structural analogy with arginine. Pentosidine is a well characterized AGE-product, formed by interaction of lysine, arginine and a reducing sugar. In vitro evidence points out towards creatine as a modifier in this reaction (Miyazaki et al., 2002). Creatine could interact in this mechanism by its amidinogroup, preventing intramolecular cross links between adjoining amino acids in proteins. In this regard, aminoguanidine is a well known inhibitor of AGE-formation (Heidland et al., 2001). Theoretically, creatine could have the same inhibiting properties on AGE-formation.

CHAPTER 5

References

Thirty spokes meet at a nave;
Because of the hole we may use the wheel.
Clay is moulded into a vessel;
Because of the hollow we may use the cup.
Walls are built around a hearth;
Because of the doors we may use the house.
Thus tools come from what exists,
But use from what does not.
Tao Te King 11/81
Lao Tse

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SAMENVATTING

In dit proefschrift werd het effect van creatinesupplementatie bij nierfalen en veroudering bestudeerd. Drie aspecten met betrekking tot het creatinemetabolisme werden in het bijzonder bestudeerd bij uremie.

Ten eerste werd de veiligheid van creatinesupplementatie bij verminderde nierfunctie nagegaan. In een uremisch diermodel werden geen effecten van creatine vastgesteld op de glomerulaire filtratie, bepaald door middel van inuline- en creatinineklaring, en op eiwitexcretie. Enkel een verhoogde serum creatinineconcentratie werd vastgesteld. Creatine vertoonde eveneens een analytische interferentie met de creatininebepaling volgens Jaffé. Hogere creatinineconcentraties na inname van creatine zijn een weerspiegeling van een hogere creatine-creatinine conversie, samen met een hogere meetwaarde door interferentie.

Ten tweede werd het effect van creatinesupplementatie op de plasma homocysteïneconcentraties bij uremie onderzocht. Verhoogde plasma homocysteïneconcentraties zijn geassocieerd met verhoogde cardiovasculaire morbiditeit en mortaliteit, vooral bij patiënten met nierlijden. Foliumzuur supplementatie is de standaard behandeling. Het is echter slechts ten dele effectief en 80 % van de patiënten met nierlijden houden verhoogde plasma homocysteïneconcentraties. De endogene synthese van creatine is gekoppeld aan de vorming van homocysteïne. Deze synthese geschiedt in twee stappen. Vooreerst wordt guanidinoacetaat gevormd uit glycine en arginine. Dit guanidinoacetaat wordt vervolgens gemethyleerd tot creatine. Deze methylatie geschiedt door methyldonatie van S-adenosylmethionine met vorming van S-adenosylhomocysteïne en vervolgens homocysteïne. Quantitatief betekent dit dat ongeveer 70% van alle *in vivo* methylaties, met methionine-homocysteïne cycli gebeuren omwille van creatinesynthese.
In het uremisch diermodel werd een significante verlaging (22%; $p<0.05$) van de plasma homocysteïneconcentraties vastgesteld bij de creatinegesupplementeerde uremische dieren, vergeleken met de uremische controle dieet dieren. De plasma homocysteïne concentraties correleerden omgekeerd met de plasma creatine concentraties. Plasma folaat en de hepatische tetrahydrofolaatconcentraties waren hoger in de uremische gesupplementeerde dieren. In een tweede experiment werd creatine als mogelijke behandeling voor hyperhomocysteinemie onderzocht in patiënten met terminaal nierfalen. Deze studie onderzocht bij 49 dialysepatiënten, reeds behandeld met foliumzuur, vitamine B12 en B6-preparaten, het effect van creatinesupplementatie op plasma

homocysteïneconcentraties in een placebo-gecontroleerde en overkruiste studie. In tegenstelling met de bevindingen in het diermodel werd bij deze dialysepatiënten geen effect van creatine op de plasma homocysteïneconcentraties vastgesteld. De creatineconcentraties na behandeling met creatine waren duidelijk toegenomen, wijzend op een adequate opname van het creatinepreparaat. In tegenstelling tot de proefdieren werden de dialysepatiënten reeds behandeld met suprafysiologische folaat, vitamine B6 en B12 preparaten. Het effect van creatine op het homocysteïnemetabolisme kan hierdoor geminimaliseerd zijn. Er werden geen negatieve effecten van creatinesupplementatie vastgesteld op de KT/V of andere parameters.

Het derde aspect betrof het inspanningsbevorderend effect van creatine. Creatine wordt als inspanningsbevorderend voedingssupplement gebruikt in verscheidene sportdisciplines. Recent werd het gebruik van creatine als therapeuticum bij patiënten met neuromusculaire aandoeningen ter verbetering van de spierfunctie onderzocht. De rationale is dat creatine inwerkt op de energiehuishouding van de cel en neuronale dood en spierdysfunctie kan verminderen.
In een cohorte van 46 oudere mannen (55-75 j) werd het effect van training, samen met creatinesupplementatie onderzocht. Na 6 maanden creatine supplementatie was er geen verschil in totale creatineconcentratie tussen placebo- en creatinegroepen, en evenmin verschillen in statische of dynamische krachtsontwikkeling van de knie-extensoren of cardiorespiratoire uithouding. Creatinesupplementatie heeft aldus geen invloed op de fysieke fitheid van deze cohorte oudere mannen.
Het effect van creatine op de myosine-isovormexpressie (myosin heavy chain (MHC)) werd nagegaan in een uremisch diermodel. In de uremische groepen werd een slow-to-fast MHC-transitie in zowel de M. soleus (verhoogd MHC IIb-gehalte) als in de M. extensor digitorum longus (hoger MHC IIb-gehalte, ten koste van MHC IIx). Creatinesupplementatie in de uremische groep, resulteerde in een lagere MHC IIb-expressie en een hogere MHC I expressie in de M. soleus. Dit werd niet vastgesteld in de M. extensor digitorum longus. Deze resultaten tonen een gewijzigde MHC-verhouding in de skeletspier bij uremie. Deze biochemische veranderingen zijn mogelijks geassocieerd met de gedaalde inspanningscapaciteit bij uremie. Creatinesupplementatie kan binnen dit diermodel de slow-to-fast overgang remmen.

SUMMARY

This thesis addresses the effects of creatine supplementation in renal failure and aging. Three aspects of creatine metabolism were studied in detail with regards to renal failure.

First, the safety of creatine supplementation in conditions with diminished renal function was investigated. No effects were observed on glomerular filtration, assessed by inulin and creatine clearance rates and on protein excretion. An apparent increase in serum creatinine concentrations was observed after creatine ingestion, together with an analytical interference of creatine on creatinine determinations by the Jafffé method. Higher creatinine concentrations after creatine ingestion are to be attributed to a higher creatine-creatinine conversion, together with an analytical interference.

Secondly, the effect of creatine supplementation on plasma homocysteine concentrations was investigated. As homocysteine is described as an important cardiovascular risk factor in renal failure patients, and folic acid treatment is only partially effective, we investigated creatine as a possible homocysteine lowering substance. Endogenous creatine synthesis is linked to the formation of homocysteine. Creatine is formed by methylation of guanidinoacetic acid through methyl donation from S-adenosylmethionine with eventually formation of homocysteine. Quantitatively this methylation process contributes to about 70% of all the methylation reactions *in vivo*.
In the uraemic animal model we observed a significant decrease in plasma homocysteine concentrations (22%; $p<0.05$) in creatine supplemented uraemic animals compared to control diet animals, and plasma homocysteine concentrations correlated inversely with plasma creatine concentrations. Plasma folate and hepatic tetrahydrofolate concentrations were higher in uraemic creatine supplemented animals. In a second experiment we investigated the use of creatine as a homocysteine-lowering substance in dialysis patients. Forty-nine patients on chronic hemodialysis, already treated with folic acid, vitamin B6 and B12 supplements were studied in a placebo-controlled cross-over study. In contrast to the findings in our animal model, we observed no effect of creatine on plasma homocysteine concentrations. Creatine concentrations were significantly increased after creatine supplementation, indicative for adequate uptake of creatine. In contrast to the animals, the patients were already treated with supraphysiological folic acid, vitamin B6 and B12 supplements, possibly masking the effect of creatine on homocysteine. No negative effects of creatine on KT/V or other parameters were observed.

The third aspect concerned the ergogenic properties of creatine. Creatine is widely used as a performance enhancing substance in several sports. Recently, the use of creatine has been investigated to improve muscular function in patients with neuromuscular disorders. Creatine could improve cellular energy metabolism, neuronal death and muscle dysfunction.
In a cohort of 46 older men (55-75y) the effect of exercise training, together with creatine supplementation was investigated. After 6 months (and 1 year (n=20)) of creatine supplementation, no difference was observed in muscular total creatine concentrations, nor in static or dynamic knee extensor force or cardiorespiratory endurance markers. In this cohort of aging man, creatine does not influence beneficially fitness.
In the uraemic animal model, the effect of creatine on the myosin heavy chain (MHC)-expression was studied. In the uraemic group, a slow-to-fast transition was observed in both M. soleus (increased MHC IIb) and in M. extensor digitorum longus (increased MHC IIb at the expense of MHCIIx). Creatine supplementation in the uraemic group resulted in a proportionally lower MHC IIb and higher MHC I-expression. This effect was not observed in the M. extensor digitorum longus. The results in this animal model show a muscle wasting with a slow-to-fast transition in uraemia. Creatine halted the slow-to-fast transition in the M. soleus.

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