

Improved reperfusion and neuroprotection by creatine in a mouse model of stroke

Konstantin Prass^{1,5}, Georg Royl^{1,5}, Ute Lindauer¹, Dorette Freyer¹, Dirk Megow¹, Ulrich Dirnagl¹, Gerda Stöckler-Ipsiroglu², Theo Wallimann³ and Josef Priller^{1,4}

¹Department of Experimental Neurology, Charité Universitätsmedizin Berlin, Berlin, Germany; ²Department of Neonatology and General Pediatrics, University Hospital of Vienna, Vienna, Austria; ³Institute of Cell Biology, ETH-Hönggerberg, Zurich, Switzerland; ⁴Department of Psychiatry and Psychotherapy, Charité Universitätsmedizin Berlin, Berlin, Germany

Stroke leads to energy failure and subsequent neuronal cell loss. Creatine and phosphocreatine constitute a cellular energy buffering and transport system, and dietary creatine supplementation was shown to protect neurons in several models of neurodegeneration. Although creatine has recently been found to reduce infarct size after cerebral ischemia in mice, the mechanisms of neuroprotection remained unclear. We provide evidence for augmented cerebral blood flow (CBF) after stroke in creatine-treated mice using a magnetic resonance imaging (MRI)-based technique of CBF measurement (flow-sensitive alternating inversion recovery-MRI). Moreover, improved vasodilatory responses were detected in isolated middle cerebral arteries obtained from creatine-treated animals. After 3 weeks of dietary creatine supplementation, minor changes in brain creatine, phosphocreatine, adenosine triphosphate, adenosine di phosphate and adenosine monophosphate levels were detected, which did not reach statistical significance. However, we found a 40% reduction in infarct volume after transient focal cerebral ischemia. Our data suggest that creatine-mediated neuroprotection can occur independent of changes in the bioenergetic status of brain tissue, but may involve improved cerebrovascular function.

Journal of Cerebral Blood Flow & Metabolism (2006) 0, 000–000. doi:10.1038/sj.jcbfm.9600351

Keywords: cerebral blood flow; cerebral ischemia; creatine; dietary supplementation; magnetic resonance imaging; neuroprotection

Introduction

Ischemic stroke results from an acute perfusion deficit leading to the disruption of metabolism, cellular energy supplies, ionic homeostasis and finally loss of cellular integrity in the infarct core. In the surrounding penumbral tissue, cerebral blood flow (CBF) is moderately reduced and a complex pathophysiologic cascade determines neuronal and glial cell fate (Dirnagl *et al*, 1999). Creatine and phosphocreatine may bolster cell survival, and

together with creatine kinase (CK) constitute a temporal and spatial buffering system for cellular energy stores. Creatine kinase can generate adenosine triphosphate (ATP) from phosphocreatine and adenosine di phosphate (ADP) at the sites of high-energy demand and restore phosphocreatine for energy storage (Wallimann *et al*, 1998). Several recent studies have shown dramatic neuroprotective effects of oral creatine administration in animal models of neurodegeneration, such as Huntington's disease (Matthews *et al*, 1998; Ferrante *et al*, 2000), Parkinson's disease (Matthews *et al*, 1999) and amyotrophic lateral sclerosis (Klivenyi *et al*, 1999). Moreover, creatine was shown to be protective in models of acute neuronal damage, such as traumatic brain injury (Sullivan *et al*, 2000) and stroke (Zhu *et al*, 2004). Creatine protected neurons from anoxia *in vitro* (Carter *et al*, 1995; Parodi *et al*, 2003) and from hypoxia–ischemia in neonatal rat brain (Adcock *et al*, 2002). In addition, creatine reduced beta-amyloid toxicity, as well as glutamate excitotoxicity, in cultured hippocampal neurons (Brewer and Wall-

Correspondence: Dr J Priller, Departments of Psychiatry and Experimental Neurology, Charité Universitätsmedizin Berlin, Schumannstrasse 20/21, D-10117 Berlin, Germany.

E-mail: josef.priller@charite.de

⁵These authors contributed equally to this work.

This work was supported in part by the Deutsche Forschungsgemeinschaft, the Hermann and Lilly Schilling Stiftung and the Swiss Society for Research on Muscle and Neuromuscular Diseases.

Received 29 January 2006; revised 24 April 2006; accepted 10 May 2006

imann, 2000). In addition, creatine improved survival and differentiation of GABA-ergic and dopaminergic neurons from human embryonic brain (Andres *et al*, 2005a,b). As a nutritional supplement, creatine is used by athletes and in patients afflicted with neuromuscular disorders (Tarnopolsky and Martin, 1999). Clinical trials attempting to assess the neuroprotective potential of creatine in neurodegenerative diseases are ongoing.

In this study, we sought to elucidate the primary target of creatine-mediated neuroprotection in an experimental model of adult stroke. While it has been suggested that creatine reduces tissue injury in the ischemic penumbra by buffering of ATP levels and inhibition of activation of caspase cell-death pathways (Zhu *et al*, 2004), these effects could be secondary and result as a consequence of improved tissue preservation in creatine-treated animals. Since it is well known that creatine crosses the blood–brain barrier with difficulty (Perasso *et al*, 2003), we hypothesized that the prophylactic effect of creatine administration in stroke may be mediated in part by improved cerebral vascular function.

Materials and methods

Male 129S6/SvEv mice 7 weeks or older were fed either an unsupplemented control diet or a diet supplemented with 0%, 5%, 1% or 2% creatine (Altromin, Germany) for the times indicated. Anhydrous creatine (purity 98%; Sigma, Germany) was titrated into the chow and pellets were generated at 55°C for <1 min. High-performance liquid chromatography analysis of the chow confirmed the concentrations of creatine and excluded conversion to creatinine. All surgical procedures were approved by the local authorities. Investigators were masked to the treatment groups.

Experimental Stroke

Middle cerebral artery occlusion (MCAO) was performed as described (Prass *et al*, 2003). Briefly, a monofilament was introduced into the left common carotid artery under halothane anesthesia, advanced to the origin of the middle cerebral artery (MCA) and left for 45 mins until reperfusion ($n=8$ to 10/group). During surgery, the rectal temperature was maintained at 37.0°C. Mice were killed after 4 days. The brains were snap-frozen. Infarct areas were quantified on hematoxylin/eosin-stained cryostat sections as described previously (Prass *et al*, 2003).

Biochemical Analysis

Measurements of creatine, phosphocreatine, ATP, ADP and adenosine monophosphate (AMP) were performed by two independent laboratories (D.M. and S.S.-I.; $n=10$ and 12). Mice were decapitated and the heads were immediately snap frozen in liquid nitrogen. The brains were removed from the skull at –20°C within 2 mins and

homogenized with ice-cold 0.5 mol/L perchloric acid containing 0.5 mmol/L ethylene glycol-bis tetraacetic acid, the acid extracts were treated as described (Ipsiroglu *et al*, 2001). High-pressure liquid chromatography was performed as described (Furst and Hallstrom, 1992).

Cell Culture

Immortalized rat brain microvascular endothelial cells (rBEC6; Blasig *et al*, 2001) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Biochrom, Germany) and 0.8% ECGF/Heparin (Promocell, Germany). At 3 days before hypoxia, cells were plated at a density of 25,000/well in the presence or absence of 2 mmol/L creatine (Sigma, Germany). Oxygen glucose deprivation (OGD) was performed for 180 mins as described (Ruscher *et al*, 2002). Creatine (2 mmol/L) was added to creatine-pretreated wells at the onset of OGD. After 1 h of reoxygenation, 3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described (Mosmann, 1983). Briefly, MTT (Sigma) was added to the cultures at a final concentration of 0.5 mmol/L. After 35 mins of incubation, 0.01 N HCl containing 5% sodium dodecyl sulfate (Sigma) was added, and optical densities were measured at a wavelength of 550 nm using a Dynatech plate reader.

Isolated Middle Cerebral Artery

Middle cerebral arteries were prepared as described (Lindauer *et al*, 2003). Mice ($n=17$ to 18/group) were anesthetized with isoflurane and decapitated. The main trunk of the MCA was dissected from the brain, cannulated on glass micropipettes in an organ chamber, and perfused continuously at a transmural pressure of 80 mm Hg. Luminal diameters were analyzed online using an inverted microscope. Vessels were allowed to equilibrate for 1 h while spontaneous tone developed, reducing the resting diameter to ~80% of the initial value. Reactivity to increased extraluminal potassium or extraluminal acidosis was examined.

Magnetic Resonance Imaging

Mice were anesthetized with 1.5% isoflurane in an oxygen/air mixture and MCAO was performed as described above. Immediately after MCAO, mice were fixed using a stereotactic frame and positioned in the magnet bore. Respiratory rate and body temperature were monitored with an MR-compatible physiology monitoring unit and temperature was maintained within physiologic limits using a heated water jacket.

MRI was performed on a Bruker 7T PharmaScan® 70/16 with a Bruker 98/38 mm RF Coil, operating on Paravision software platform (Bruker, Germany). Perfusion weighted imaging (PWI) was performed with a spin echo echo planar imaging sequence with a 180° Gauss RF inversion pulse (imaging parameters: echo time = 16.23 ms; imaging slice thickness = 2 mm; image matrix = 64 × 64; field of

view = 20.141×20 mm; inversion parameters: inversion slice thickness = 6 mm; pulse length = 1 ms) modified from published protocols of flow-sensitive alternating inversion recovery (FAIR) imaging (Detre *et al*, 1992; Kim, 1995). Five neighboring slices at 2 mm distance between olfactory bulb and cerebellum were selected. For each slice, 2×11 images were acquired for one CBF image: the first series with a slice selective inversion, the second with a nonselective inversion (Figure 4A). The first image was performed with an inversion time (TI) of 114 ms, in each of the succeeding 10 images the TI was increased by 800 ms ($TI = 114 \dots 8114$ ms). From the resulting relaxation curves, the longitudinal relaxation time constant $T1$ was obtained for the slice selective and slice nonselective inversion by fitting the data points according to $S(TI) = A + |S_0(1 - 2e^{-TI/T1})|$ with $S(TI)$ being the signal intensity after TI , A the absolute bias, S_0 the signal intensity at $TI = 0$ s (Figure 4B). For each timepoint, $T1$ was determined for slice selective inversion ($T1_{sel}$) and for slice nonselective inversion ($T1_{nonse}$) (Figure 4C). Cerebral blood flow was quantified using by $CBF = \lambda((1/T1_{sel}) - (1/T1_{nonse})/272)$ with λ being the blood-tissue water partition coefficient (assumed to be 0.9 ml/g^{26}) (Figure 4D). After each acquisition of PWI, diffusion weighted imaging (DWI) was performed in the same sample volume with a Stejskal-Tanner-like multi-slice spin echo echo planar imaging sequence (imaging parameters: echo time = 66.5 ms, $\Delta = 25$ ms, diffusion gradient duration = 5 ms, 10 slices with slice thickness 1 mm, image matrix = 128×128 , field of view = 20×20 mm, 15 averages). In each sequence, one image without diffusion weighting ($b = 0 \text{ s/mm}^2$) and one with diffusion weighting ($b = 1500 \text{ s/mm}^2$) was acquired to allow for a quantitative analysis of the apparent diffusion coefficient (ADC). The ADC was calculated using $ADC = -1/b \ln(S(b)/S_0)$ with b being the b-value, $S(b)$ the signal intensity with diffusion gradient, S_0 the signal intensity without diffusion gradient ($b = 0$). The sequence was repeated three times, each time with a different direction of the diffusion gradient (orthogonal in read, slice and phase direction), resulting in a total scan time of 20 mins. To obtain an isotropic diffusion estimate, the mean ADC of these three directions was calculated. During MCAO, one PWI (15 to 30 mins post-MCAO) and one DWI acquisition (30 to 55 mins post-MCAO) was performed. After 60 mins of MCAO, reperfusion was allowed and two more sets of PWI and DWI were obtained.

For quantitative analysis, each slice was divided into two horizontal symmetrical halves. Omitting the two most posterior 1-mm-slices in DWI and the most posterior 2-mm-slice in PWI, hemispheric ADC and CBF values of the remaining slices were averaged for each time point.

Statistics

A two-tailed Student's *t*-test (two groups) or one-way ANOVA with subsequent Bonferroni correction (>2 groups) were used. Values for $P < 0.05$ were considered statistically significant.

Results

Infarct Volumes

We examined the effects of 3 weeks of dietary creatine supplementation on infarct volumes in mice (Figure 1). Oral administration of 1% and 2% creatine resulted in a significant reduction in infarct volumes compared with control diet. We found a dose-dependent neuroprotective effect of creatine, leading to the reduction of lesion sizes by 8%, 37% and 42% when 0.5%, 1% and 2% oral creatine were administered, respectively. To determine whether creatine could also protect mice from stroke when given after the onset of ischemia, we injected 30 mg/g body weight of creatine intraperitoneally at the end of surgery. The altered route of administration was required since we found that food ingestion varied in mice after stroke. Based on previously published results (Perasso *et al*, 2003), a more than 1000-fold increase in serum creatine concentration will result from this treatment. However, this high dose of creatine did not reduce infarct sizes when administered after stroke (Figure 1).

Since the occurrence of ischemic events in patients at risk for stroke cannot be anticipated, long-term oral creatine supplementation would be required. We therefore administered 2% oral creatine or control diet to mice for 12 months and subjected them to 45 mins of MCAO. Surprisingly, the protective effect of creatine was abolished and no differences in infarct volumes were observed (control: $60.9 \text{ mm}^3 \pm 37$ versus creatine: $58.4 \text{ mm}^3 \pm 35$).

Brain Energy Metabolites

We presumed that the neuroprotective effects of creatine in stroke would be a function of the

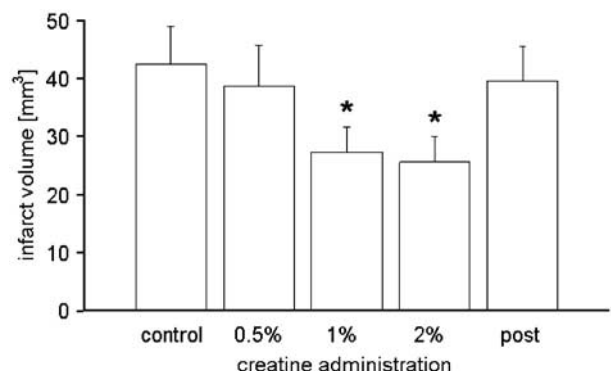


Figure 1 Reduced infarct size after oral creatine administration. Infarct volumes after 45 mins of MCA occlusion in mice fed for 3 weeks with control diet (control) or diet supplemented with 0.5%, 1% and 2% creatine. Post-treatment (post) of mice with a single dose of creatine i.p. (30 mg/g body weight) after MCAO. Data are presented as means + s.d. ($n = 8$ to 10/group). * indicates statistical significance between control and creatine-administered groups at $P < 0.05$ (ANOVA with Bonferroni's correction).

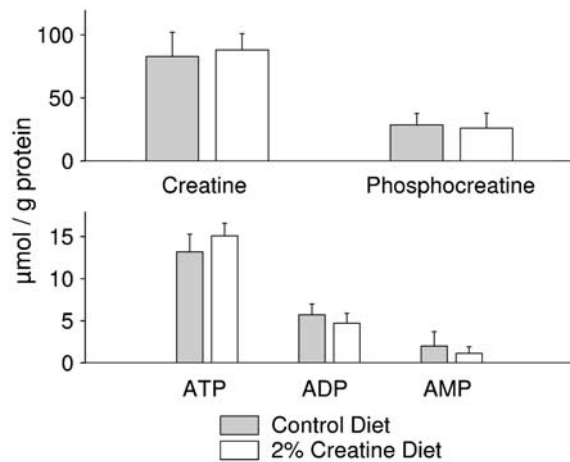


Figure 2 Bioenergetic status of brain tissue is not altered by creatine administration. Concentrations of creatine, phosphocreatine and energy-rich phosphates in the brains of mice fed for 3 weeks with control diet ($n = 10$, gray bars) and diet supplemented with 2% creatine ($n = 10$, white bars). The data are presented in $\mu\text{mol/g}$ protein as means \pm s.d. No significant differences in metabolite levels were detected between the groups (unpaired two-tailed Student's t -test).

concentration of energy metabolites in the brain. In studies performed in two independent laboratories (DM and SS-I), total creatine, creatine, phosphocreatine and ATP concentrations in brain were found to be similar, irrespective of whether the animals had received control or 2% creatine-supplemented diet for 3 weeks (Figure 2). The creatine/phosphocreatine ratio approximated 3:1. Absolute ATP concentrations and the ratios of ATP/ADP and ATP/AMP were not significantly different between both groups, and provided no evidence for run down of high-energy phosphates. HPLC-analysis of the chow confirmed the concentrations of creatine and excluded conversion to creatinine (data not shown).

In the setting of cerebral ischemia, phosphocreatine can help restore depleted ATP levels through the activity of CK. Thus, the neuroprotective effects of oral creatine administration could also be mediated by the modulation of CK activity in the brain. We measured CK activities 24 and 72 h after cerebral ischemia in the ischemic and nonischemic hemispheres of mice fed control or 2% creatine-enriched diet. No significant differences in CK activities were found between both groups (data not shown).

Vascular Function

Since creatine did not cross the blood–brain barrier in our model, we hypothesized that its neuroprotective effect could result from improved cerebral vascular function. In immortalized brain microvascular endothelial cells, treatment with 2 mmol/L creatine for 3 days significantly reduced MTT cleavage by viable cells (Gerlier and Thomasset,

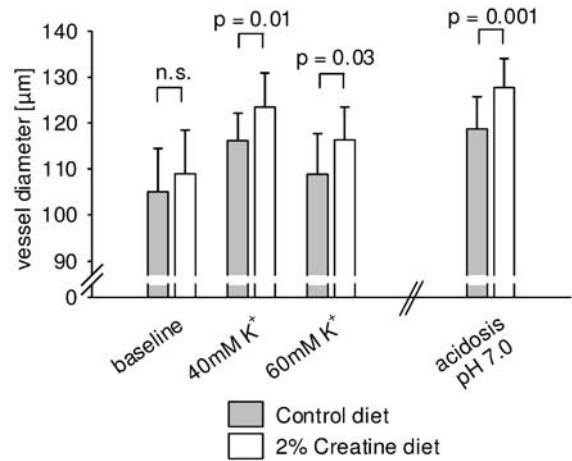


Figure 3 Improved vasodilatory responses in middle cerebral arteries from creatine-treated mice. Absolute diameters of isolated MCA derived from mice fed for 3 weeks with control diet ($n = 18$, gray bars) and diet supplemented with 2% creatine ($n = 17$, white bars) in response to increased extraluminal potassium (40, 60 mmol/L) and acidosis (pH 7.0). The data are presented as means \pm s.d. Under baseline conditions, mean vessel diameters in creatine-fed mice were not significantly different (NS) compared with controls. All other conditions showed significantly higher mean vessel diameters in creatine-fed mice compared with controls (P -values from unpaired two-tailed Student's t -test with Bonferroni's correction).

1986) after oxygen-glucose deprivation (control: OD 510.5 ± 69.9 creatine-treated: OD 469.4 ± 65.8 ; $P = 0.03$) without affecting the cell number (data not shown). Vascular reactivity of isolated MCA was examined after 3 weeks of 2% creatine versus control diet (Figure 3). After equilibration, spontaneous tone developed and reduced vessel diameters to 109 ± 10 and $105 \pm 9 \mu\text{m}$ in the arteries from creatine-treated and control mice, respectively ($P = 0.23$). The dilation in response to elevated extraluminal potassium concentrations of 40 and 60 mmol/L was significantly increased in arteries from creatine-treated compared with control animals ($P = 0.01$ and 0.03 , respectively). Moreover, the dilatory response of isolated MCA to acidosis was also significantly enhanced ($P = 0.001$). In contrast to non-cerebral vessels or the basilar or posterior cerebral artery, middle cerebral arteries only constrict in response to very high concentrations of extraluminal potassium (hypertonic, > 80 mmol/L), and dilate in response to concentrations up to 70 to 80 mmol/L, as shown previously for rats (Golding et al, 2000).

In vivo Magnetic Resonance Imaging Measurement of Perfusion and Diffusion

To assess the functional consequences of altered vascular reactivity by creatine, we performed *in vivo* MRI measurements of CBF and tissue damage during ischemia and reperfusion. Respiratory rate

and rectal temperature were kept within physiologic limits in creatine-treated and control mice (Table 1). We developed a novel technique of *in vivo* CBF measurement using FAIR-MRI in mice as described in detail under Material and methods (Figures 4A to 4D). Comparison of CBF values obtained using FAIR-MRI with autoradiographic analysis of CBF in mice revealed matching results (Leithner *et al*, unpublished observations). During MCAO, DWI (Figure 4E) and PWI (Figure 4F) showed a pronounced reduction in the ischemic MCA territory. For quantitative analysis, DW and PW images were averaged across each hemisphere. During acute ischemia, ADC and CBF decreased significantly ($P < 0.000001$) in the ischemic compared with the nonischemic hemispheres of both creatine-treated and control animals. The reductions in ADC or CBF did not differ between treatment and control groups, suggesting that the severity of ischemia was comparable at the time of analysis (data not shown). However, creatine-treated mice recovered faster from ischemia: 15 to 35 mins after reperfusion, mean CBF was significantly higher in creatine-treated mice compared with controls ($P = 0.01$). After 61 to 81 mins of reperfusion, the difference in CBF values between the groups was not significant ($P = 0.18$). Interestingly, ADC recovery in creatine-treated mice lagged behind CBF recovery: 35 to 55 mins after reperfusion, only a trend towards higher ADC was observed in the treatment group ($P = 0.21$), whereas ADC was significantly higher in the ischemic hemispheres of creatine-treated mice compared with controls after 75 to 95 mins of reperfusion ($P = 0.01$) (Figure 5).

Discussion

In this study, we found that short-term dietary (1% to 2%) creatine supplementation for 3 weeks dose-dependently reduced stroke size in mice. These findings are in line with recently published results using a more severe MCAO model in mice (Zhu *et al*, 2004). All studies found that creatine needed to be administered for weeks before stroke onset to be effective. Based on the fact that creatine crosses the blood-brain barrier poorly (Perasso *et al*, 2003), it is

not surprising that 1 week of dietary creatine administration failed to protect mice from stroke and that even after 4 weeks of creatine-enriched diet, no increase in creatine concentration was observed in the nonischemic brain (Zhu *et al*, 2004). We also did not detect significant changes in the bioenergetic status of brain tissue (levels of creatine, phosphocreatine and ATP) after 3 weeks of dietary creatine supplementation in mice. It should be noted that a comprehensive analysis of the pharmacokinetics of oral supplementation of creatine-monohydrate in different species revealed comparatively poor uptake of creatine into mouse brain, albeit a 32% increase in total brain creatine concentration was observed when mice were fed with creatine for 8 weeks (Ipsiroglu *et al*, 2001).

In addition to most of the previous studies that have linked neuroprotection with increased levels of creatine and/or phosphocreatine in brain (Matthews *et al*, 1998; Klivenyi *et al*, 1999; Matthews *et al*, 1999; Ferrante *et al*, 2000; Sullivan *et al*, 2000), we observed beneficial creatine effects in stroke that did not directly relate to the concentrations of creatine and energy-rich phosphates in the brain. These findings do not diminish the role of creatine as a buffering system for cellular energy stores, but suggest that creatine may have additional neuroprotective properties. Thus, creatine was recently reported to afford cytoprotection via direct antioxidant activity (Sestili *et al*, 2006). Moreover, creatine was found to act in concert with mitochondrial CK in inhibiting permeability transition pore opening, an early trigger of apoptosis (Dolder *et al*, 2003). While it has been suggested that creatine buffers ATP levels and inhibits the activation of caspase cell-death pathways in the striatal ischemic core (Zhu *et al*, 2004), these effects could result from improved tissue preservation in creatine-treated animals. In fact, ATP loss can be used to differentiate infarct core from energetically compromised penumbral tissue after stroke (Hata *et al*, 2000). It is therefore tempting to speculate that the prophylactic effect of creatine administration in stroke is not mediated primarily by buffering of ATP levels.

Instead, we propose that creatine may be taken up by vascular endothelial and/or smooth muscle cells without measurable contribution to total brain

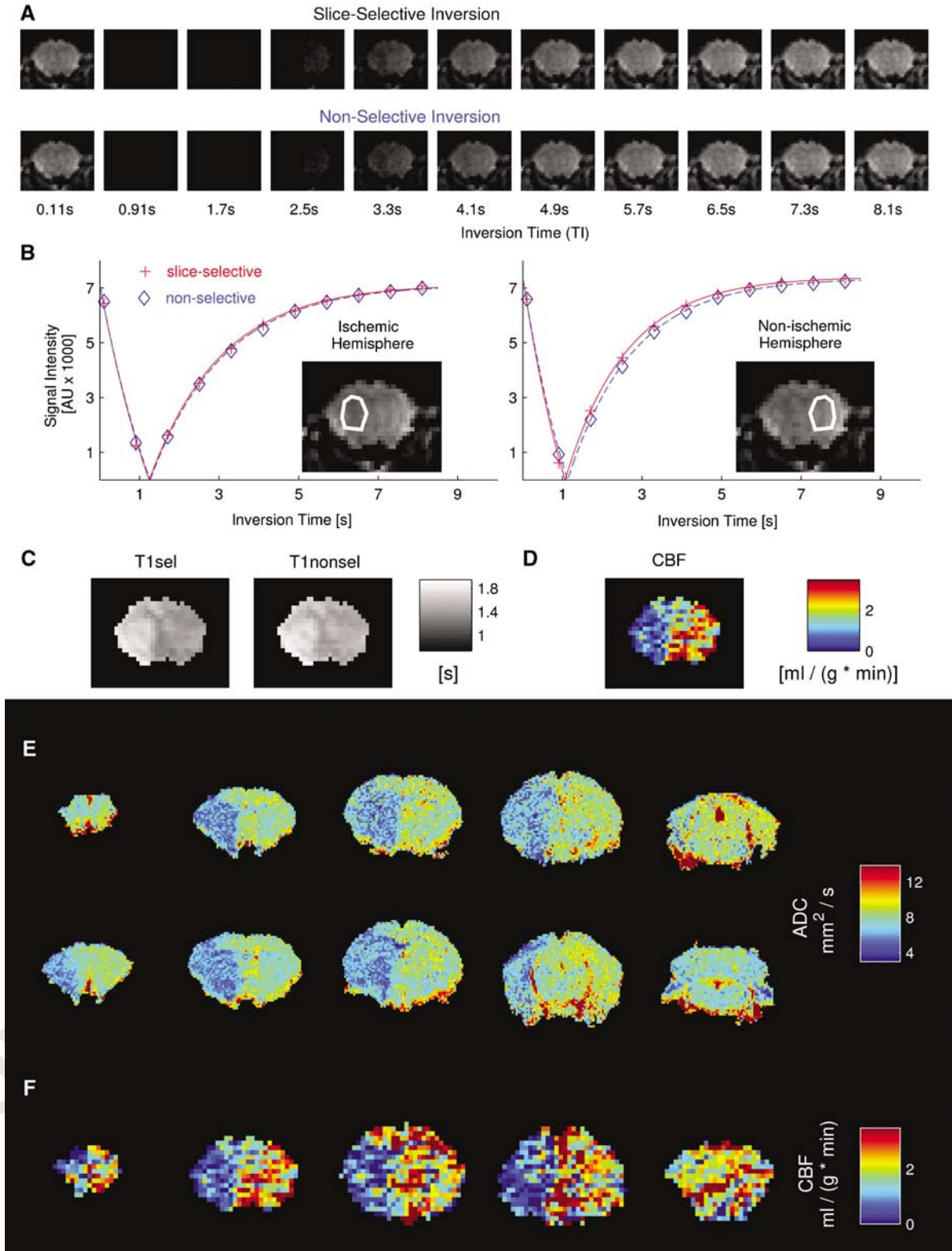
Table 1 Physiological parameters during MRI

Measurement	Creatine	Control	Creatine	Control
PWI 15–35 mins post-MCAO	105 ± 17	115 ± 13	37.6 ± 0.3	37.5 ± 0.6
DWI 35–55 mins post-MCAO	105 ± 21	120 ± 16	37.8 ± 0.6	37.9 ± 0.6
PWI 15–35 mins postreperfusion	100 ± 14	83 ± 16	38.0 ± 0.5	37.5 ± 0.9
DWI 35–55 mins postreperfusion	104 ± 23	86 ± 14	38.1 ± 0.4	37.8 ± 0.4
PWI 55–75 mins postreperfusion	97 ± 17	79 ± 19	38.0 ± 0.4	38.0 ± 0.8
DWI 75–95 mins postreperfusion	99 ± 17	83 ± 23	38.3 ± 0.4	38.3 ± 0.8

Respiratory rate and body temperature of mice treated with creatine ($n = 6$) and controls ($n = 6$) were monitored during MRI. Alternating measurements of perfusion weighted imaging (PWI) and diffusion weighted imaging (DWI) were performed. One set of PWI/DWI was obtained during ischemia (15–55 mins after MCAO), two sets were obtained during reperfusion (15–95 mins of reperfusion). Data are presented as means ± s.d.

creatine concentration at 3 weeks of dietary supplementation. Both vascular endothelial cells (Decking *et al*, 2001) and smooth muscle cells (Ishida *et al*, 1991) contain creatine, as well as cytosolic and mitochondrial isoforms of CK. In support of our

hypothesis, we found that creatine prevents the activation of brain endothelial cells in response to OGD. Moreover, *ex vivo* vascular reactivity to vasodilatory stimuli, which are pathophysiologically relevant in stroke, such as acidosis and



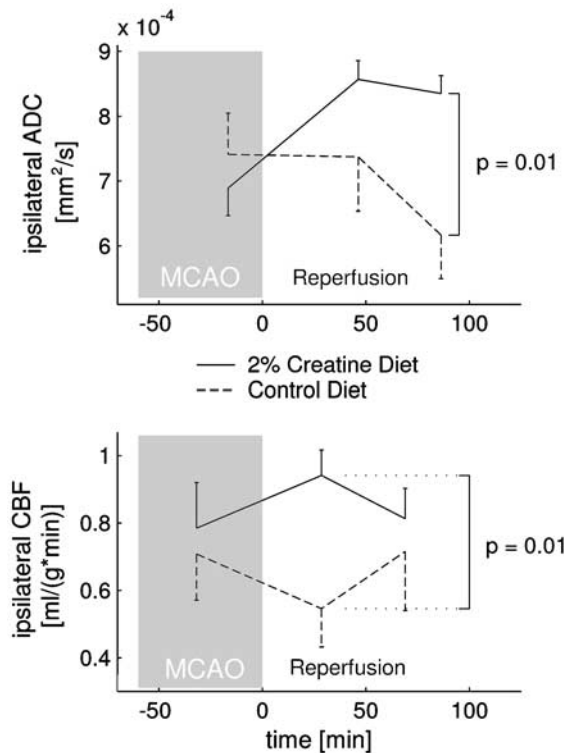


Figure 5 Reduced stroke size as a result of accelerated restoration of cerebral blood flow in creatine-fed mice. Diffusion weighted imaging of ADC (upper panel) and PWI/FAIR-MRI analysis of CBF (lower panel) during 60 mins of MCAO and up to 90 mins of reperfusion in mice fed for 3 weeks with control diet ($n = 6$, dashed line) and diet supplemented with 2% creatine ($n = 6$, solid line). Time courses of ADC and CBF in the ischemic hemispheres expressed as means \pm s.e.m. Where differences between groups are significant, P -values from unpaired two-tailed Student's t -test with Bonferroni's correction are shown.

increased extracellular potassium concentrations, is improved in response to creatine. This could be mediated by Ca^{2+} -activated and/or ATP-sensitive potassium channels, which are known to be involved in vasodilatory responses to hypoxia/ischemia, acidosis and 40 mmol/L potassium (Reid and Paterson, 1996; Lindauer *et al*, 2003). Interestingly, sodium/potassium ATPase and ATP-sensitive potassium channels were recently found to be functionally coupled to CK (Guerrero *et al*, 1997; Selivanov *et al*, 2004).

Figure 4 *In vivo* MRI measurement of perfusion and diffusion during and after cerebral ischemia. Representative PWI/FAIR-MRI analysis (A–D: single 2 mm brain slice 15 mins post-MCAO, F: series of caudo-frontal brain slices) and diffusion weighted imaging (E: series of fronto-caudal brain slices) during left-sided MCAO in a mouse. (A) In a single slice, 22 consecutive images were acquired with an inversion recovery sequence. The first 11 images follow slice selective inversion, the second 11 images nonselective inversion. The inversion time TI was varied between 0.1 and 8.1 s. (B) Signal intensities plotted against TI and fitted TI relaxation curve in two selected regions of interest in the ischemic (left panel) and the nonischemic (right panel) hemispheres; slice-selective inversion (red) and nonselective inversion (blue). (C) Maps of calculated TI for slice selective inversion (TI_{sel}) and nonselective inversion (TI_{nonrel}). (D) CBF map obtained with $\text{CBF} = \lambda (1/TI_{\text{sel}} - 1/TI_{\text{nonrel}})$ with λ (blood–tissue water partition coefficient) assumed to be 0.9 ml/g. (E) Diffusion weighted imaging shows ADC reduction in the ischemic hemisphere at 30 to 45 mins post-MCAO. (F) PWI reveals CBF reduction in the ischemic hemisphere at 15 to 30 mins post-MCAO.

To assess whether oral creatine administration also improves vascular function *in vivo*, we wanted to monitor CBF and ADC in the same animal during cerebral ischemia and reperfusion. To this end, we used MRI and adapted a FAIR-based technique of quantitative CBF measurement to mice. In the past, FAIR-MRI was used in rats (Detre *et al*, 1992; Tsekos *et al*, 1998) and gerbils (Pell *et al*, 1999). Recently, FAIR-MRI has also been used to investigate global CBF changes in a model of sickle cell disease (Kennan *et al*, 2004). To our knowledge, this is the first study that employs FAIR as a tool to monitor acute CBF impairment in a mouse model of stroke, opening the possibility of using this technique in future studies involving knockout mice. Interestingly, we found that creatine-treated animals recovered significantly faster from cerebral ischemia compared with controls. Notably, CBF was increased during reperfusion and tissue diffusion recovered faster as a result of creatine administration, which is more likely to result from direct effects of creatine on the vascular system as suggested by our *ex vivo* and *in vitro* data than increased demand by surviving neurons as a result of neuroprotection.

Our findings may provide a novel additional mechanism of creatine-induced neuroprotection and suggest that creatine improves CBF after stroke by preserving vascular cell function. Interestingly, we found that life-long creatine administration failed to protect adult mice from stroke, suggesting that adaptive mechanisms could compromise the beneficial roles of creatine. This needs to be taken into consideration when creatine is to be employed long-term as a neuroprotective agent in patients afflicted with neuromuscular or neurodegenerative disorders. It would be interesting to determine whether the CBF changes we observed with short-term creatine administration persist with prolonged treatment.

References

- Adcock KH, Nedelcu J, Loenneker T, Martin E, Wallimann T, Wagner BP (2002) Neuroprotection of creatine supplementation in neonatal rats with transient cerebral hypoxia–ischemia. *Dev Neurosci* 24:328–82
- Andres RH, Ducray AD, Huber AW, Perez-Bouza A, Krebs SH, Schlattner U, Seiler RW, Wallimann T, Widmer HR

Q3

- (2005a) Effects of creatine treatment on survival and differentiation of GABA-ergic neurons in cultured striatal tissue. *J Neurochem* 95:33–45
- Andres RH, Huber AW, Schlattner U, Perez-Bouza A, Krebs SH, Seiler RW, Wallimann T, Widmer HR (2005b) Effects of creatine treatment on the survival of dopaminergic neurons in cultured fetal ventral mesencephalic tissue. *Neuroscience* 133:701–13
- Blasig IE, Giese H, Schroeter ML, Sporbert A, Utepbergenov DI, Buchwalow IB, Neubert K, Schonfelder G, Freyer D, Schimke I, Siems WE, Paul M, Haseloff RF, Blasig R (2001) NO and oxyradical metabolism in new cell lines of rat brain capillary endothelial cells forming the blood–brain barrier. *Microvasc Res* 62:114–27
- Brewer GJ, Wallimann TW (2000) Protective effect of the energy precursor creatine against toxicity of glutamate and beta-amyloid in rat hippocampal neurons. *J Neurochem* 74:1968–78
- Carter AJ, Muller RE, Pschorn U, Stransky W (1995) Preincubation with creatine enhances levels of creatine phosphate and prevents anoxic damage in rat hippocampal slices. *J Neurochem* 64:2691–9
- Decking UK, Alves C, Wallimann T, Wyss M, Schrader J (2001) Functional aspects of creatine kinase isoenzymes in endothelial cells. *Am J Physiol Cell Physiol* 281:C320–8
- Detre JA, Leigh JS, Williams DS, Koretsky AP (1992) Perfusion imaging. *Magn Reson Med* 23:37–45
- Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* 22:391–7
- Dolder M, Walzel B, Speer O, Schlattner U, Wallimann T (2003) Inhibition of the mitochondrial permeability transition by creatine kinase substrates. Requirement for microcompartmentation. *J Biol Chem* 278:17760–6
- Ferrante RJ, Andreassen OA, Jenkins BG, Dedeoglu A, Kuemmerle S, Kubilus JK, Kaddurah-Daouk R, Hersch SM, Beal MF (2000) Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. *J Neurosci* 20:4389–97
- Furst W, Hallstrom S (1992) Simultaneous determination of myocardial nucleotides, nucleosides, purine bases and creatine phosphate by ion-pair high-performance liquid chromatography. *J Chromatogr* 578:39–44
- Gerlier D, Thomasset N (1986) Use of MTT colorimetric assay to measure cell activation. *J Immunol Methods* 20:57–63
- Golding EM, Steenberg ML, Johnson TD, Bryan RM, Jr (2000) The effects of potassium on the rat middle cerebral artery. *Brain Res* 880:159–66
- Guerrero ML, Beron J, Spindler B, Groscurth P, Wallimann T, Verrey F (1997) Metabolic support of Na⁺ pump in apically permeabilized A6 kidney cell epithelia: role of creatine kinase. *Am J Physiol* 272:C697–706
- Hata R, Maeda K, Hermann D, Mies G, Hossmann KA (2000) Dynamics of regional brain metabolism and gene expression after middle cerebral artery occlusion in mice. *J Cereb Blood Flow Metab* 20:306–15
- Herscovitch P, Raichle ME (1985) What is the correct value for the brain–blood partition coefficient for water? *J Cereb Blood Flow Metab* 5:65–9
- Ipsiroglu OS, Stromberger C, Ilas J, Hoyer H, Muhl A, Stockler-Ipsiroglu S (2001) Changes of tissue creatine concentrations upon oral supplementation of creatine-monohydrate in various animal species. *Life Sci* 69:1805–15
- Ishida Y, Wyss M, Hemmer W, Wallimann T (1991) Identification of creatine kinase isoenzymes in the guinea-pig. Presence of mitochondrial creatine kinase in smooth muscle. *FEBS Lett* 283:37–43
- Kennan RP, Suzuka SM, Nagel RL, Fabry ME (2004) Decreased cerebral perfusion correlates with increased BOLD hyperoxia response in transgenic mouse models of sickle cell disease. *Magn Reson Med* 51:525–32
- Kim SG (1995) Quantification of relative cerebral blood flow change by flow-sensitive alternating inversion recovery (FAIR) technique: application to functional mapping. *Magn Reson Med* 34:293–301
- Klivenyi P, Ferrante RJ, Matthews RT, Bogdanov MB, Klein AM, Andreassen OA, Mueller G, Wermer M, Kaddurah-Daouk R, Beal MF (1999) Neuroprotective effects of creatine in a transgenic animal model of amyotrophic lateral sclerosis. *Nat Med* 5:347–50
- Lindauer U, Vogt J, Schuh-Hofer S, Dreier JP, Dirnagl U (2003) Cerebrovascular vasodilation to extraluminal acidosis occurs via combined activation of ATP-sensitive and Ca²⁺-activated potassium channels. *J Cereb Blood Flow Metab* 23:1227–38
- Matthews RT, Ferrante RJ, Klivenyi P, Yang L, Klein AM, Mueller G, Kaddurah-Daouk R, Beal MF (1999) Creatine and cyclocreatine attenuate MPTP neurotoxicity. *Exp Neurol* 157:142–9
- Matthews RT, Yang L, Jenkins BG, Ferrante RJ, Rosen BR, Kaddurah-Daouk R, Beal MF (1998) Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. *J Neurosci* 18:156–63
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63
- Parodi M, Rebaudo R, Perasso L, Gandolfo C, Cupello A, Balestrino M (2003) Effects of exogenous creatine on population spike amplitude and on postanoxic hyperexcitability in brain slices. *Brain Res* 963:197–202
- Pell GS, Lythgoe MF, Thomas DL, Calamante F, King MD, Gadian DG, Ordidge RJ (1999) Reperfusion in a gerbil model of forebrain ischemia using serial magnetic resonance FAIR perfusion imaging. *Stroke* 30:1263–70
- Perasso L, Cupello A, Lunardi GL (2003) Kinetics of creatine in blood and brain after intraperitoneal injection in the rat. *Brain Res* 974:37–42
- Prass K, Scharff A, Ruscher K, Lowl D, Muselmann C, Victorov I, Kapinya K, Dirnagl U, Meisel A (2003) Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin. *Stroke* 34:1981–6
- Reid JM, Paterson DJ (1996) Role of K⁺ in regulating hypoxic cerebral blood flow in the rat: effect of glibenclamide and ouabain. *Am J Physiol* 270:H45–52
- Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, Sawitzki B, Priller J, Dirnagl U, Meisel A (2002) Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an *in vitro* model. *J Neurosci* 22:10291–301
- Selivanov VA, Alekseev AE, Hodgson DM, Dzeja PP, Terzic A (2004) Nucleotide-gated KATP channels integrated with creatine and adenylate kinases: amplification, tuning and sensing of energetic signals in the compartmentalized cellular environment. *Mol Cell Biochem* 256–257:243–56
- Sestili P, Martinelli C, Bravi G, Piccoli G, Curci R, Battistelli M, Falcieri E, Agostini D, Gioacchini AM, Stocchi V (2006) Creatine supplementation affords cytoprotection in oxidatively injured cultured mamma-

- lian cells via direct antioxidant activity. *Free Radical Biol Med* 40:837–49
- Sullivan PG, Geiger JD, Mattson MP, Scheff SW (2000) Dietary supplement creatine protects against traumatic brain injury. *Ann Neurol* 48:723–9
- Tarnopolsky M, Martin J (1999) Creatine monohydrate increases strength in patients with neuromuscular disease. *Neurology* 52:854–7
- Tsekos NV, Zhang F, Merkle H, Nagayama M, Iadecola C, Kim SG (1998) Quantitative measurements of cerebral blood flow in rats using the FAIR technique: correlation with previous iodoantipyrine autoradiographic studies. *Magn Reson Med* 39:564–73
- Wallimann T, Dolder M, Schlattner UM, Hornemann T, O’Gorman E, Ruck A, Brdiczka D (1998) Some new aspects of creatine kinase (CK): compartmentation, structure, function and regulation for cellular and mitochondrial bioenergetics and physiology. *Biofactors* 8:229–34
- Zhu S, Li M, Figueroa BE, Liu A, Stavrovskaya IG, Pasinelli P, Beal MF, Brown RH, Jr, Kristal BS, Ferrante RJ, Friedlander RM (2004) Prophylactic creatine administration mediates neuroprotection in cerebral ischemia in mice. *J Neurosci* 24:5909–12

UNCORRECTED PROOF