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Attenuation by creatine of myocardial metabolic stress in Brattleboro rats caused by chronic inhibition of nitric oxide synthase

[1]Dumitru Constantin-Teodosiu, Paul L. Greenhaff, Sheila M. Gardiner, Michael D. Randall, Julie E. March & Terence Bennett

Department of Physiology and Pharmacology, University Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 The present experiment was undertaken to investigate: (a) the effect of nitric oxide synthase (NOS) inhibition, mediated by oral supplementation of the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), on measures of myocardial energy metabolism and function; (b) the effect of oral creatine supplementation on these variables, in the absence and presence of L-NAME.

2 In one series of experiments, 4 weeks oral administration of L-NAME (0.05 mg ml^{-1} day^{-1} in the drinking water) to Brattleboro rats caused significant reductions in myocardial ATP, creatine, and total creatine concentrations and an accumulation of tissue lactate when compared with control animals. Administration of creatine (0.63 mg ml^{-1} day^{-1} in the drinking water) for 4 weeks elevated myocardial creatine and total creatine concentrations and reduced lactate accumulation, but did not significantly affect ATP or phosphocreatine (PCr). Concurrent treatment with creatine and L-NAME prevented the reduction in creatine and total creatine concentrations, and significantly attenuated the accumulation of lactate and the reduction in ATP seen with L-NAME alone.

3 In a second series of experiments, 4 weeks treatment with L-NAME and creatine plus L-NAME increased mean arterial blood pressure in conscious Brattleboro rats. Hearts isolated from these animals showed decreased coronary flow and left ventricular developed pressure (LVDP), and total mechanical performance. Treatment with creatine alone had no measurable effect on either mean arterial blood pressure or coronary flow in isolated hearts. However, there was an increase in LVDP, but not in total mechanical performance, because there was a bradycardia.

4 These results indicate that creatine supplementation can attenuate the metabolic stress associated with L-NAME administration and that this effect occurs as a consequence of the action of creatine on myocardial energy metabolism.

Keywords: L-NAME; ATP; lactate; muscle; creatine; NO synthase; Brattleboro rats

Introduction

Myocardial high energy phosphate metabolism has been shown to be abnormal in conditions such as left ventricular hypertrophy and congestive heart failure (Conway *et al.*, 1991), and is usually manifested as a decrease in the phosphocreatine (PCr) to ATP ratio. One of the major causes of these conditions is systemic arterial hypertension (Kannel *et al.*, 1987), and there is some evidence for alterations in cardiac energy metabolism during development of genetic hypertension (Lortet *et al.*, 1993). Recently, it has been found that chronic inhibition of nitric oxide synthase (NOS) by prolonged oral administration of NOS inhibitors causes sustained hypertension (Gardiner *et al.*, 1992; 1993). Since there are no data regarding myocardial energy metabolism in this novel model of hypertension, the first objective of the present study was to measure indices of myocardial energy metabolism in animals treated for 4 weeks with the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) (Gardiner *et al.*, 1990; 1992; 1993).

Oral supplementation of creatine has been shown to increase skeletal muscle creatine concentration by an average of 20% in man (Harris *et al.*, 1992). More recently, dietary creatine supplementation has been shown to reduce the extent of skeletal muscle ATP loss, and plasma ammonia and hypoxanthine accumulation (accepted markers of metabolic energy crisis), during maximal skeletal muscle contraction in man (Balsom *et al.*, 1993; Greenhaff *et al.*, 1993; 1994b). Reduced creatine availability has been implicated in the metabolic abnormalities observed in hypertrophied human myocardium (Ingwall *et al.*, 1985; Conway *et al.*, 1991). Since we found chronic treatment with L-NAME caused decreases in cardiac ATP and creatine concentrations (see Results), our second objective was to determine the effect of oral creatine supplementation on myocardial energy metabolism and function, in the absence and presence of chronic L-NAME treatment. A preliminary account of this work has been given to the British Pharmacological Society (Constantin-Teodosiu *et al.*, 1994).

Methods

Two series of experiments (A and B) were performed on male, homozygous Brattleboro (i.e. vasopressin-deficient) rats (4-6 months old). These animals are polydipsic, and hence addition of substances to their drinking water ensures they receive sustained dosing (Gardiner *et al.*, 1990; 1992; 1993). Four groups of animals were studied in both experiments:

Group 1: animals drinking tap water for 4 weeks ($n=13$ experiment A, $n=7$ experiment B).

Group 2: animals drinking tap water containing L-NAME.HCl (Sigma, U.K.) (0.05 mg ml^{-1} day^{-1}, 10-12 mg day^{-1}) for 4 weeks ($n=7$ experiment A, $n=7$ experiment B).

[1] Author for correspondence.

Group 3: animals drinking tap water containing creatine (Cairn Chemicals, U.K.) (0.63 mg ml^{-1} day^{-1}, 126–158 mg day^{-1}) for 4 weeks ($n=6$ experiment A, $n=8$ experiment B).

Group 4: animals drinking tap water containing L-NAME (0.05 mg ml^{-1} day^{-1}, 10–12 mg day^{-1}) and creatine (0.63 mg ml^{-1} day^{-1}, 126–158 mg day^{-1}) for 4 weeks ($n=7$ experiment A, $n=6$ experiment B).

The L-NAME dose was chosen because it has previously been demonstrated to cause sustained hypertension in Brattleboro rats (Gardiner *et al.*, 1990; 1992; 1993). The dose of creatine administered was, following adjustment for body weight, similar to that which has previously been shown to increase markedly skeletal muscle creatine concentration in man (Harris *et al.*, 1992). Fluid intake (200–250 ml day^{-1}) was not influenced by the presence of L-NAME and/or creatine in the water.

Experiment A

At the end of the experimental period, animals were anaesthetized (sodium methohexitone, 40–60 mg kg^{-1} body wt., i.p.) and their hearts were rapidly removed and snap-frozen in liquid nitrogen. Subsequently, tissues were freeze-dried and ATP, PCr, creatine and lactate concentrations were measured in sections of the ventricular apex (Harris *et al.*, 1974). Total creatine was calculated as the sum of PCr and creatine.

Experiment B

Four to five weeks after the onset of oral supplementation, animals were anaesthetized (sodium methohexitone, 40–60 mg kg^{-1} body wt., i.p.) and had an intra-arterial catheter implanted in the distal abdominal aorta via the ventral caudal artery for the measurement of mean arterial blood pressure, and an intravenous catheter implanted in the jugular vein (Gardiner *et al.*, 1990). Arterial blood pressure was recorded over a period of 30 min when animals were fully conscious and freely moving, and at least 24 h after catheter implantation. The technique has been described in detail by Gardiner *et al.* (1990).

Following measurement of blood pressure, *in vitro* cardiac function was assessed as follows. All animals were heparinized (1000 u kg^{-1} body wt., i.v.) and anaesthetized with sodium pentobarbitone (44 mg kg^{-1} body wt., i.v.) (Sagatal, Rhône Mérieux, Harlow, Essex, U.K.). In each case, following a thoracotomy, the heart was rapidly excised and placed in ice cold oxygenated Krebs-Henseleit solution (containing (mmol l^{-1}): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2 and glucose 10) to arrest cardiac contraction. The aortic stump was then cannulated and the heart perfused retrogradely, according to the method of Langendorff (1895), at a constant pressure of 60 mmHg with oxygenated Krebs-Henseleit buffer. A water-filled latex balloon catheter, coupled to a pressure transducer, was inserted through the pulmonary vein and advanced into the left ventricle in order to measure left ventricular developed pressure (LVDP). In each case, left ventricular end diastolic pressure was initially set at 5 mmHg by adjusting the volume of fluid in the balloon. The pressure transducer was coupled to a MacLab 4e recording system (AD Instruments, New South Wales, Australia), and heart rate was derived from the pressure signal. Coronary flow was measured by means of a transit time ultrasonic flow meter (model T106, Transonic Systems Incorporated, Ithaca, New York, U.S.A.) coupled to an extracorporeal flow probe placed in series with the aortic cannula.

In experiments A and B, pooled data were analysed in the first instance by analysis of variance (ANOVA). When a significant difference was observed, differences between treatments were located by use of Student's t test for unpaired data (two-tailed); a P value <0.05 was taken as significant; values are means ± s.e.mean.

Results

Experiment A

Treatment with L-NAME for 4 weeks caused significant reductions in myocardial ATP, creatine and total creatine con-

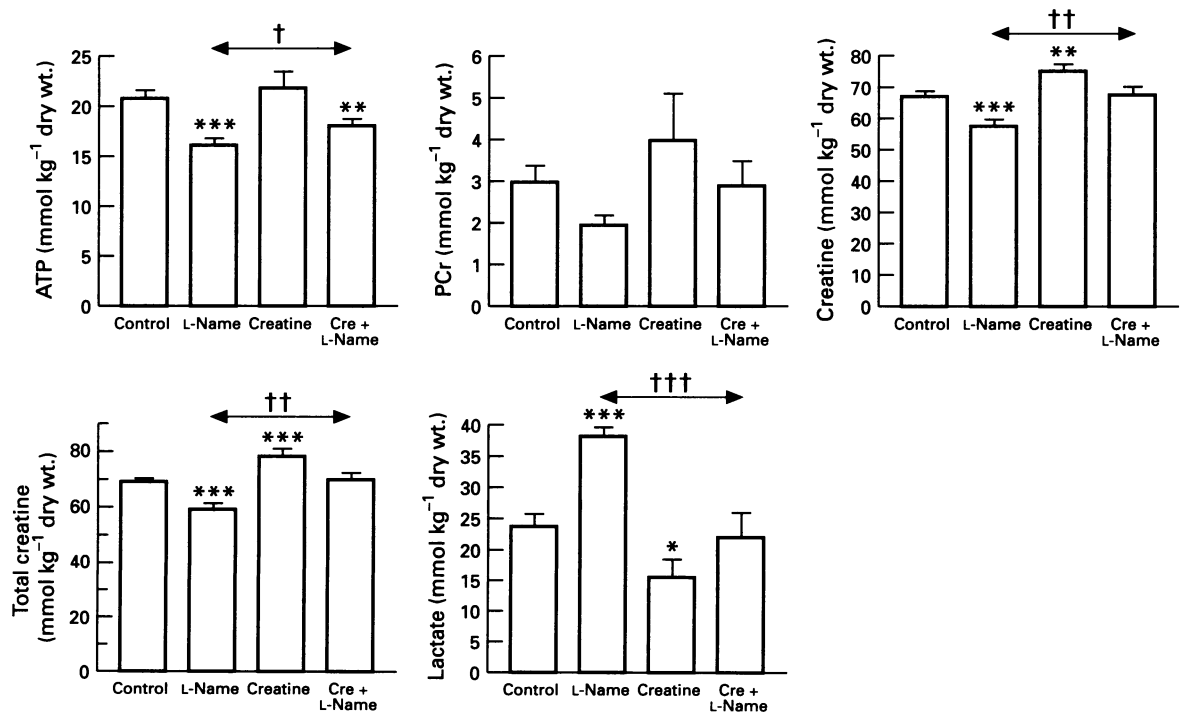

Figure 1 Ventricular heart muscle concentrations of ATP, phosphocreatine, creatine, total creatine and lactate in control ($n=13$), L-NAME ($n=7$), creatine ($n=6$) and creatine plus L-NAME ($n=7$)-treated rats. Values are means ± s.e.mean. Significantly different from controls: $*P<0.05$; $**P<0.01$; $***P<0.001$. Significant difference between L-NAME and creatine plus L-NAME-treated rats: $\dagger P<0.05$; $\dagger\dagger P<0.01$; $\dagger\dagger\dagger P<0.001$.

centrations, and a marked increase in tissue lactate concentration (Figure 1). Administration of creatine alone for 4 weeks increased creatine and total creatine concentrations in the heart, and reduced lactate accumulation, but did not significantly affect ATP or PCr (Figure 1). Concurrent treatment with creatine and L-NAME prevented the reduction in creatine and total creatine concentrations, and significantly attenuated the accumulation of lactate and the reduction in ATP, seen with L-NAME alone (Figure 1).

Experiment B

The mean arterial blood pressure recorded for each experimental group is shown in Table 1. Treatment with L-NAME for 4 weeks caused a 42% increase in mean arterial blood pressure. Treatment with creatine alone had no measurable effect on blood pressure, and treatment with creatine and L-NAME produced an increase blood pressure similar to that recorded with L-NAME alone. The treatment regimens did not influence heart rate *in vivo* (Table 1).

The effects of the experimental treatments on cardiac function *in vitro* are shown in Table 2. In the control preparations, coronary flow, either absolute or corrected for dry mass, was not different from that in the preparations from animals receiving creatine alone, but was significantly greater than that in the hearts from rats treated with L-NAME, either alone or in combination with creatine. The LVDP was greater in the hearts from creatine-treated rats compared to controls (94.6 ± 5.2 v. 72.0 ± 7.7 mmHg), but this was accompanied by significant bradycardia in the creatine-fed group. Hence, the total mechanical performance, assessed in terms of the pressure-rate product, did not differ between controls and those receiving creatine. In contrast, the mechanical performances of the hearts from the groups receiving L-NAME, and L-NAME plus creatine, were significantly depressed relative to the appropriate controls (Table 2). In both cases, LVDP was reduced and there was significant bradycardia compared to the control preparations. In the hearts from rats treated with L-NAME, the concomitant administration of creatine did not result in any improvement in mechanical performance or LVDP (Table 2).

Discussion

The results of the present study clearly show that chronic administration of L-NAME caused abnormalities in myocardial energy metabolism. The changes observed in lactate and PCr concentrations indicate that the contribution of anaerobic energy delivery to total energy production was greater in these animals, suggesting that the energy demand of contraction was greater, and/or that oxygen delivery or extraction was reduced. The lower myocardial ATP concentration indicates that the relative degree of metabolic stress, and the subsequent adenine nucleotide loss, were greater in L-NAME-treated animals and is representative of what would be expected to occur during local tissue ischaemia. In accordance with this, Table 2 shows that the metabolic changes observed in hearts from animals treated with L-NAME were associated with a 37% decrease in coronary flow.

In the present work we studied polydipsic Brattleboro rats in order to ensure that they received constant dosing with the substances added to their drinking water. Elsewhere (Batin *et al.*, 1991) we have shown that *in vivo* myocardial function in Brattleboro rats is not different from that of control, Long Evans rats. Hence, there is no reason to suppose that the experimentally induced changes in myocardial metabolism would be confined to Brattleboro rats. Moreover, the absence of reflex bradycardia in the presence of the elevated blood pressure seen in the animals given L-NAME or creatine plus L-NAME, probably reflected adaptation of the baroreflex, since acute exposure to L-NAME does cause bradycardia which wanes with time (Gardiner *et al.*, 1993). The substantial reduction in coronary flow observed following chronic L-NAME and creatine plus L-NAME treatments is consistent with the well established role of nitric oxide in the regulation of coronary vascular tone (Amezcua *et al.*, 1989). It is likely that the relative myocardial ischaemia resulting from this reduction in coronary flow may well have resulted in the reduced cardiac mechanical performance, as evidenced by the reduction in LVDP and the bradycardia. The bradycardia observed *in vitro* contrasts the lack of effect on heart rate *in vivo* and this may perhaps be explained by the absence of compensatory sympathetic drive in the *in vitro* preparations.

Table 1 Mean arterial blood pressures and heart rate in conscious Brattleboro rats in Experiment B

	Control ($n=7$)	*L-NAME* ($n=6$)	*Creatine* ($n=8$)	*Creatine + L-NAME* ($n=5$)
Blood pressure (mmHg)	114 ± 3	162 ± 7***	113 ± 6	183 ± 9***†††
Heart rate (beats min^{-1})	361 ± 14	380 ± 12	357 ± 17	374 ± 14

Values are mean ± s.e.mean. Significantly different from the control group ***$P<0.001$; significant difference between creatine and creatine plus L-NAME-treated rats †††$P<0.001$.

Table 2 Cardiac variables from the isolated buffer-perfused hearts in Experiment B

	Control ($n=7$)	*L-NAME* ($n=7$)	*Creatine* ($n=7$)	*Creatine + L-NAME* ($n=6$)
Coronary flow (ml min^{-1})	14.8 ± 0.8	9.3 ± 1.4**	15.8 ± 0.6	7.3 ± 0.7***†††
LVDP (mmHg)	72.0 ± 7.7	38.9 ± 8.3*	94.9 ± 5.2*	40.4 ± 5.4**†††
Heart rate (beats min^{-1})	294 ± 13	236 ± 71**	241 ± 92**	199 ± 8***††
Pressure-rate product (mmHg min^{-1})	$20,777 \pm 1,836$	$9,351 \pm 2,191$**	$22,691 \pm 1,172$	$8,161 \pm 1,348$***†††

Values are means ± s.e.mean. Significantly different from the control group. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Significant difference between creatine and creatine plus L-NAME-treated rats; ††$P<0.01$, †††$P<0.001$.

Figure 1 demonstrates that creatine supplementation was successful in ameliorating the metabolic dysfunction caused by L-NAME. Furthermore, it would appear that this effect was achieved independently of an influence of L-NAME on coronary blood flow, cardiac function, and/or hypertension, since in the group of rats treated with creatine plus L-NAME, coronary flow, mechanical function and blood pressure were still abnormal (Tables 1 and 2). The ability of creatine to reverse the metabolically stressful effects of L-NAME is consistent with the reported beneficial effect of creatine on skeletal muscle energy metabolism during maximal contraction (Balsom *et al.*, 1993; Greenhaff *et al.*, 1993; 1994b) and recovery from ischaemic contraction (Greenhaff *et al.*, 1994a), and is in agreement with the growing body of evidence suggesting that creatine availability may play a critical role in the control of mitochondrial respiration in both skeletal (Bessman & Fonyo, 1966) and cardiac tissue (Field *et al.*, 1994). Indeed, the findings of the present study demonstrate that, following creatine supplementation alone, *in vitro* LVDP was increased in parallel with a reduction in myocardial lactate accumulation and heart rate, whilst at the same time blood pressure, coronary flow and pressure-rate product remained normal. This suggests that creatine supplementation may favourably affect both myocardial function and metabolism. However, it should be noted that there was a dissociation between improvements in myocardial function and metabolism following creatine plus L-NAME supplementation. Furthermore, despite some restoration of myocardial ATP following creatine plus L-NAME, this process was not complete. This suggests that a higher dose of creatine supplementation may result in an improvement in both metabolism and function in L-NAME-treated animals. We would like to postulate that the favourable metabolic effects associated with creatine supplementation may have occurred because the free creatine concentration of the cytosol was increased to a level sufficient to stimulate mitochondrial ATP production. This suggestion is supported by recently published data demonstrating that creatine supplementation can accelerate the rate of mitochondrial linked PCr resynthesis in human skeletal muscle during recovery from intense ischaemic muscular contraction (Greenhaff *et al.*, 1994). In accordance with this, the present work showed that the mean myocardial PCr concentration was higher than normal following creatine supplementation. The role of creatine as an acceptor of mitochondrial ATP (the creatine shuttle) has been discussed previously in a series of papers (Bessman & Fonyo, 1966; Bessman & Geiger, 1982; Meyer *et al.*, 1984; Walliman *et al.*, 1992). The present results go against reports indicating that a marked reduction in intracellular creatine concentration, caused by feeding the creatine analogue, β-guanidinopropionic acid (β-GPA), has no effect on contractile function and/or ATP production, during steady-state contraction in skeletal (Shoubridge & Radda, 1984), or cardiac muscle (Shoubridge *et al.*, 1985). But the interpretation of results from those studies is open to some debate, due to the concomitant effects that β-GPA is known to have on tissue structure (Shields *et al.*, 1975; Laskowski *et al.*, 1981).

It could be suggested that the differences in myocardial lactate concentration between treatments in the present experiment occurred as a result of L-NAME and creatine influencing skeletal muscle energy metabolism, which subsequently influenced myocardial lactate as a consequence of blood contamination. However, this explanation seems unlikely as, firstly, L-NAME and creatine have been found to have no effect on skeletal muscle lactate in this model (unpublished data), and, secondly, the changes observed in myocardial ATP are consistent with the view that the lactate concentrations recorded were representative of myocardial energy metabolism.

The uptake of creatine by the myocardium during creatine supplementation was less than might have been expected, based on the degree of uptake observed in human skeletal muscle (Harris *et al.*, 1992; Greenhaff *et al.*, 1994a). However, uptake was greater than in previous studies, where creatine was added to rat chow (Osbakken *et al.*, 1992), and was still sufficient to counter the adverse metabolic effects of L-NAME in the present work. The reduction in total creatine following L-NAME administration (Figure 1) was unexpected, but suggests that L-NAME could have influenced creatine biosynthesis and/or myocardial creatine uptake and release. An interaction between L-NAME and creatine metabolism is feasible, considering that L-arginine is the precursor for creatine and for nitric oxide synthesis. Indeed, we cannot refute the argument that the changes observed in energy metabolism in the present study were, at least partly, a consequence of L-NAME inhibiting creatine synthesis, and/or myocardial creatine uptake and release. This question could be addressed by investigating the effects of creatine supplementation on myocardial function and metabolism in another experimental model of ischaemia.

Thus, in conclusion, the present data indicate that chronic ingestion of creatine attenuates the metabolic abnormalities produced in cardiac tissue by L-NAME administration and that this effect occurs as a direct consequence of the action of creatine on myocardial energy metabolism. The present findings point to a role for creatine supplementation in the treatment of conditions associated with myocardial dysfunction.

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