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ORIGINAL CONTRIBUTION

Effects of creatine in a rat intestinal model of ischemia/reperfusion injury

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Abstract
Purpose Creatine belongs to a buffering system of cellular ATP level and has been reported to display direct antioxidant activity. Aim of this work was to investigate whether creatine treatment could ameliorate the antioxidant response of intestinal cells and limit the oxidative injury induced by anoxia and subsequent reoxygenation.
Methods Jejunal and ileal tracts of rat intestine were everted and incubated in vitro under normoxic, anoxic and reoxygenation conditions in the absence and in the presence of 10 mM creatine. (Na^+, K^+)-ATPase, γ-GT and antioxidant enzymes activities were determined in mucosal homogenate, as well as malondialdehyde production and HSP70 expression.
Results Both in jejunum and ileum, creatine treatment increases (Na^+, K^+)-ATPase activity; γ-GT is unaffected in jejunum but stimulated in ileum. In both tissues, creatine does not alter the antioxidant activities or malondialdehyde level. HSP70 expression is increased only in jejunum. Anoxic conditions stimulate antioxidant activities to a greater extent in jejunum compared to ileum; reoxygenation does not evoke further effects, but enhances malondialdehyde production in both tracts. The protective action of creatine, in reoxygenation, is more marked in jejunum as for its stimulation of antioxidant activities; however, in jejunum, a prooxidant action of creatine is suggested, since malondialdehyde production is enhanced by its presence; on the contrary in ileum, where HSP70 is overexpressed in reoxygenation, peroxidation level is significantly reduced.
Conclusions The presence of creatine seems to potentiate the defensive response of both tissues, in jejunum by means of cell antioxidant equipment, in ileum by the involvement of HSP70.

Keywords Creatine · Rat · Jejunum · Ileum · Ischemia/reperfusion

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Introduction

Creatine (Cr, N-[aminoiminomethyl]-N-methyl glycine) is an endogenous amino acid produced from glycine, methionine and arginine in the liver, kidney and pancreas [1]; in mammals, creatine is also obtained from the diet by meat-containing products [2, 3]. In both cases, creatine is transported via the blood to the tissues, where the uptake into the cells is carried out by a specific transporter (CT) that facilitates the entry of creatine against a very large concentration gradient [4–6].

Diet intake, in normal conditions, supplies about 50% of creatine requirement [2, 3]; besides as ergogenic aid to improve athletes exercise performance, creatine dietary supplement is increasingly used as a possible therapeutic agent in the treatment of various muscle, neurological and neuromuscular disease conditions [7–10]. Actually, the potential of creatine to be protective has been illustrated in numerous models of neurodegeneration as well as in

animal models of traumatic brain injury and cerebral ischemia [9, 11].

The putative benefits of creatine in these disorders have been generally attributed to the creatine-induced buffering of cellular ATP levels, whose fall would stimulate the formation of reactive oxygen species (ROS) and tissue oxidative damage [3]. Indeed, most of these pathologies recognize multiple etiological factors, among which the detrimental role of oxidative stress has been stably recognized [12–14]. Moreover, it has been reported that creatine displays direct antioxidant activity both in acellular [15] and cellular systems [16, 17].

Massive ROS production was identified as an important causative factor for ischemia and reperfusion (I/R), that can lead to tissue injury, and are serious complications in organ transplantation, myocardial infarction and stroke [18, 19]. Previous research supports the hypothesis that the intestine is most sensitive to I/R injury among the other internal organs [19]; indeed, although the gastrointestinal tract represents only about 5% of body weight, it is responsible for about 20% of whole-body oxygen consumption [20]. A marked zonation of mitochondrial density, enzymes and other proteins along the small intestinal epithelial cells was evidenced [21], suggesting a difference in transporting nutrients and responding to I/R-induced damage among intestinal tracts.

Due to the antioxidative properties of creatine, it seems possible that using creatine may protect the intestinal tissue against oxidative I/R injury. In the present study, we therefore tried to determine whether creatine could ameliorate the antioxidant response of intestinal cells and prevent intestinal tissue injury induced by an in vitro model of I/R, namely anoxia and subsequent reoxygenation. To the best of our knowledge, there has not been such a report in the literature. To reach this goal, we measured various oxidant and antioxidant parameters, both in isolated rat jejunum and ileum, under normoxia, anoxia and reoxygenation conditions in the absence and in the presence of creatine.

Methods

Everted sac preparation

Male albino rats (Wistar strain, Charles River Italiana) weighing 250–300 g (about 2 months age), fed on a rodent laboratory chow and tap water, were used. The rats were deeply anaesthetized with an injection of 2,2,2-tribromoethanol [0.05 g (250 g body weight)$^{-1}$ I.P.] and then decapitated. The experiments were performed according to national ethical guidelines and approved by "Comune di Milano—Uff. Diritti degli animali", "Regione Lombardia" and "Ministero della Salute" (prot. 5/2008, approved on November 2008, validity 3 years).

Two segments of jejunum were removed, everted, incubated and perfused for 60 min at 28 °C. This temperature was chosen since at the body temperature, at least in our hands, the transport activity of the "in vitro" intestine does not seem to be in a steady state [22]. The mucosal side of the intestine was incubated in 15 ml of Krebs-Henseleit-Tris solution at pH 7.5 (composition in mM: 140.0 Na^+, 5.9 K^+, 2.5 Ca^{2+}, 1.2 Mg^{2+}, 127.7 Cl^-, 12.2 SO_4^{2-}, 25.0 Hepes/Tris) added with 5.56 mM D-glucose and, when requested, with 10 mM creatine. The serosal side of the intestine was incubated in 1 ml of the same solution lacking glucose and creatine. Unless indicated differently, the mucosal bathing solution was constantly bubbled with 100% O_2; during episodes of anoxia, we switched to 100% N_2.

After 5 min preincubation, the 60 min experimental period started. Anoxic conditions were imposed by bubbling the mucosal solution with 100% N_2 for 15 min after 45 min with 100% O_2. Reoxygenation was obtained by bubbling the mucosal solution with 100% O_2 for 45 min after 15 min with 100% N_2.

At the end of the experiment, the intestine was blotted on filter paper and weighed. The mucosal layer was scraped off at 0 °C and immediately weighed. A part of the scraped mucosa was used for Western blot experiments. The remaining mucosa was resuspended in 50 mM phosphate buffer, pH 7.4 (10% w/v), homogenized and used for γ-glutamyltransferase (γ-GT) and (Na^+, K^+)-ATPase determinations. The protein content was determined by the method of Bradford [23] using bovine serum albumin as the standard. The remaining homogenate was centrifuged at 10,000×g for 10 min at 4 °C in a refrigerated centrifuge (Hermle, Germany). The supernatant so obtained was used for the assays of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). For the estimation of lipid peroxidation by means of malondialdehyde (MDA) determination, homogenate was prepared in chilled 1.15% (w/v) KCl solution.

Enzyme activities

All enzyme activities were expressed as mU/mg proteins and in the figures are reported in percent over the control.

(Na^+, K^+)-ATPase. The (Na^+, K^+)-ATPase activity was estimated by a slightly modified method of Schöner et al. [24] in which the resynthesis of the ATP split by the ATPase is coupled to NADH oxidation via pyruvate kinase-lactate dehydrogenase reaction.

γ-GT. γ-GT was measured according to Persijn and Van der Slik [25] by using a commercial kit (Sentinel Diagnostics, Milano, Italy).

LDH. LDH was determined by using a commercial kit (Sentinel Diagnostics, Milano, Italy).

Antioxidant enzymes

CAT. CAT activity was measured according to the method of Aebi [26] by following the decrease in absorbance of H_2O_2 at 240 nm for 5 min.

SOD. SOD activity was measured by the inhibition of pyrogallol autoxidation at 420 nm according to Guzik et al. [27].

GPx. GPx activity was measured by following the oxidation of NADPH at 340 nm according to Anwer et al. [28].

GR. GR activity was measured as a decrease in absorbance of NADPH for 5 min at 340 nm according to Ojano-Dirain et al. [29].

Lipid peroxidation

MDA production, expressed as nmol/mg proteins, was assessed spectrophotometrically with the method defined by Ohkawa et al. [30]. In the figures, MDA production is reported in percent over the control.

Protein extraction and Western blot

After the everted sac experiments previously described, the jejunal mucosa was scraped off and total proteins were extracted. Briefly, the scraped material from each rat was resuspended in cold buffer sucrose-histidine (IS) containing 0.3 M sucrose, 25 mM Histidine, 1 mM EDTA, supplemented with protease inhibitors (Roche), homogenized and then centrifuged at 4 °C for 15 min at 5,000×*g*. Supernatant was recovered; protein concentration was measured [23], and 60 μg proteins were analyzed by 7% SDS–PAGE electrophoresis. The proteins transferred to a polyvinylidene difluoride membrane (Miniprotean 3, Biorad) were probed overnight at 4 °C with the specific primary antibodies. Anti HSPA1A-Heat shock protein 70 kDa protein 1A (Aviva) diluted 1:5000 in 5% nonfat dry milk-TBST buffer was used. The primary antibody for HSP70 was detected with a goat anti-rabbit IgG conjugated to horseradish peroxidase (Chemicon) used at 1:40000 dilution in 5% nonfat dry milk-TBST buffer. Site of antibody–antigen reaction was visualized by using Amersham ECL Plusfollowed by autoradiography.

Statistics

Statistical analysis was done by Student's t test or by analysis of variance (ANOVA) followed by posthoc Tukey's limitation. Values reported in the text are means ± S.E.

Results

Effects of creatine on (Na^+, K^+)-ATPase, antioxidant enzyme activities and MDA production in jejunal and ileal mucosal homogenates

The current study demonstrates that in both rat jejunum and ileum, acute creatine treatment exerts a stimulating effect on (Na^+, K^+)-ATPase activity, suggesting protective properties for creatine. In the presence of creatine, γ-GT activity is unaffected in the jejunum but significantly stimulated, over the control, in the ileum. In both intestinal tissues, creatine does not alter the activities of SOD, CAT, GPx and GR compared to their corresponding controls, nor MDA level, index of the status of lipid peroxidation (Tables 1, 2).

Table 1 Effects of 10 mM creatine on (Na^+, K^+)-ATPase, γ-GT, antioxidant enzyme activities and MDA production of jejunal mucosal homogenate

	Creatine (percent over control)	*P*
(Na^+, K^+)-ATPase	+87 ± 4.4	<0.01
γ-GT	+31 ± 22	n.s.
SOD	–8 ± 12	n.s.
CAT	+15 ± 21	n.s.
GPx	+60 ± 47	n.s.
GR	–2 ± 22	n.s.
MDA	+0.2 ± 21	n.s.

Values are means ± S.E. Number of experiments = 5 with duplicate estimation

Table 2 Effects of 10 mM creatine on (Na^+, K^+)-ATPase, γ-GT, antioxidant enzyme activities and MDA production of ileal mucosal homogenate

	Creatine (percent over control)	*P*
(Na^+, K^+)-ATPase	+77 ± 13	<0.01
γ-GT	+37 ± 10	<0.05
SOD	+38 ± 29	n.s.
CAT	+22 ± 19	n.s.
GPx	+35 ± 30	n.s.
GR	+23 ± 25	n.s.
MDA	–28.5 ± 13	n.s.

Values are means ± S.E. Number of experiments = 5 with duplicate estimation

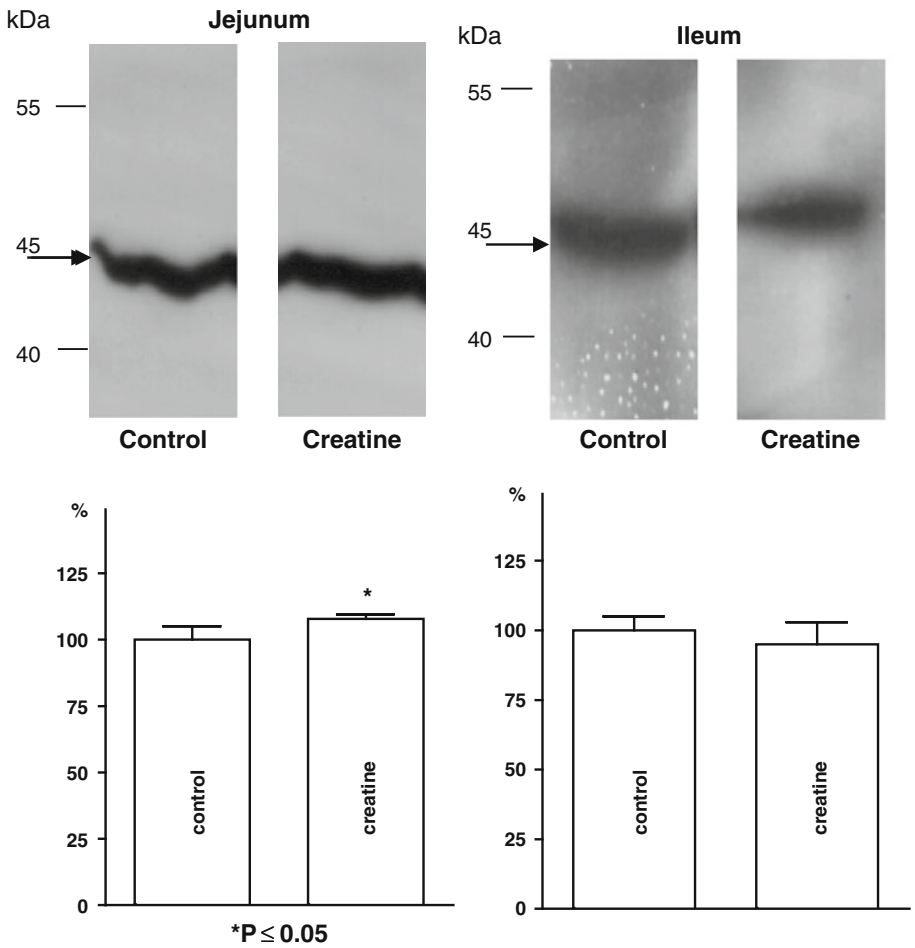


Fig. 1 Effects of 10 mM creatine on HSP70 expression in jejunal and ileal mucosal homogenates. Western blot analysis for HSP70 was performed on jejunal and ileal total proteins and was carried out on 4 experiments. Densitometric analysis of the bands reveals significant statistical difference in the intensity of the signals only for jejunum

Effects of creatine on HSP70 expression in jejunal and ileal mucosal homogenates

HSP70 confers stress tolerance and cytoprotection against several environmental-induced injury conditions [31, 32]; thus, the possible induction of HSP70 by creatine was investigated. HSP70 protein expression was measured using Western blotting in homogenates obtained after everted sac experiments carried out in the presence of 10 mM creatine. Assays, performed on jejunal and ileal total proteins (Fig. 1), revealed the presence of a band at the specific kDa value for HSP70. HSP70 protein level is markedly increased in the jejunum by the presence of creatine and densitometric analysis of the band reveals significant statistical difference in the intensity of the signal with respect to the control. On the contrary, creatine does not influence HSP70 expression in the ileal intestinal tract.

Effects of anoxia, reoxygenation and creatine on antioxidant enzyme activities of jejunal and ileal mucosal homogenates

As shown in Fig. 2, jejunal mucosa reacts to anoxic injury: as a matter of fact, SOD, GPx and GR activities are significantly increased, while CAT activity is reduced in anoxic conditions with respect to the control. When anoxia is followed by 45 min reoxygenation, a significant reduction in GPx is apparent. These results agree with data reported in the literature [18, 19, 33] and confirm the involvement of ROS in I/R conditions. In the jejunal tract, the presence of creatine markedly stimulates all antioxidant activities tested with respect to reoxygenation conditions.

The antioxidant response of ileum is less pronounced (Fig. 3). SOD and GR activities increase in anoxia, but no changes are evident for GPx and CAT. After reoxygenation, SOD is significantly reduced. Creatine presence is not so effective either, if compared to the jejunum, since it leads to an increase in GR and to a reduction in CAT and GPx activities with respect to reoxygenation conditions.

Effects of anoxia, reoxygenation and creatine on (Na^+, K^+)-ATPase, γ-GT and MDA production in jejunal and ileal mucosal homogenates

Figure 4 indicates that in jejunum anoxic conditions reduce (Na^+, K^+)-ATPase activity, as expected since blockage of the oxygen supply causes a decrease in intracellular ATP

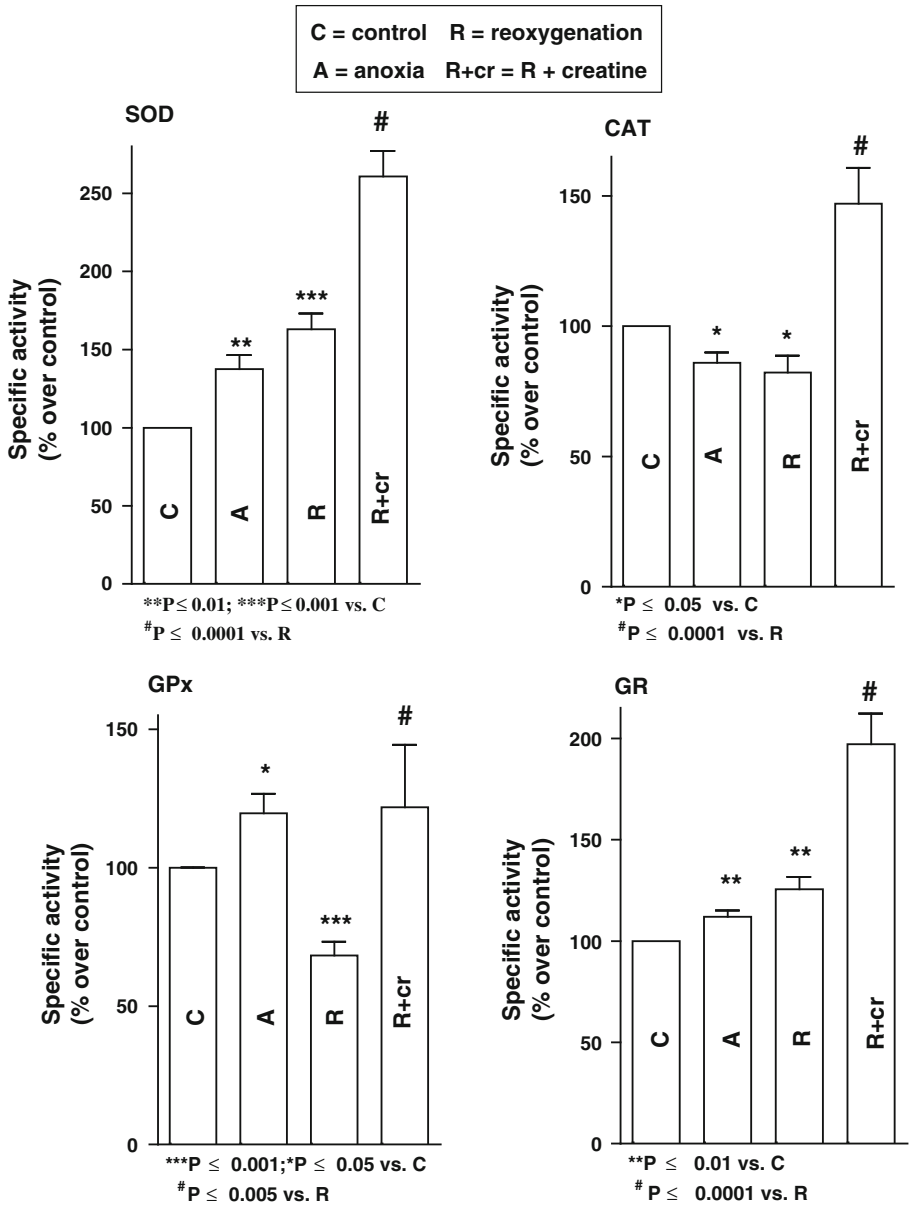


Fig. 2 Effects of anoxia/reoxygenation and 10 mM creatine on antioxidant enzyme activities of jejunal mucosal homogenate. Values are means ± S.E. Number of experiments = 5 with duplicate estimation

reserves. On the contrary, γ-GT activity is stimulated: this result, in combination with GPx and GR enhancement shown in Fig. 2, indicates that the GSH redox cycle may have a crucial role in the anoxia-induced intestinal response. In the meantime, a lowered MDA production is apparent, consistent with both the reduced aerobic metabolism and the sustained antioxidant response. As a matter of fact, reoxygenation markedly increases (Na^+, K^+)-ATPase activity and MDA level. The presence of creatine in the latter condition significantly stimulates both (Na^+, K^+)-ATPase and γ-GT activities; however, MDA production increases dramatically in the presence of creatine, suggesting that acute creatine treatment may favor a prooxidant state in the jejunum. Thus, creatine appears to have both cytoprotective effect, owing to the stimulation of antioxidant enzymes and (Na^+, K^+)-ATPase activity, and cytotoxic effect caused by the increase in lipid peroxidation.

The pattern of responses of ileum to anoxia/reoxygenation overlaps the ones evidenced in the jejunum as for γ-GT activity and MDA production, while (Na^+, K^+)-ATPase activity is unaffacted by the imposed conditions (Fig. 5). The presence of creatine stimulates (Na^+, K^+)-ATPase activity with respect to the reoxygenation condition, but significantly reduces both γ-GT and MDA. Thus, since apparently creatine reduces lipid peroxidation in ileum, the prooxidant effect observed in the jejunum seems to be absent.

To evaluate the possibility that creatine affects biological state of the intestinal cells, we measured leakage of the

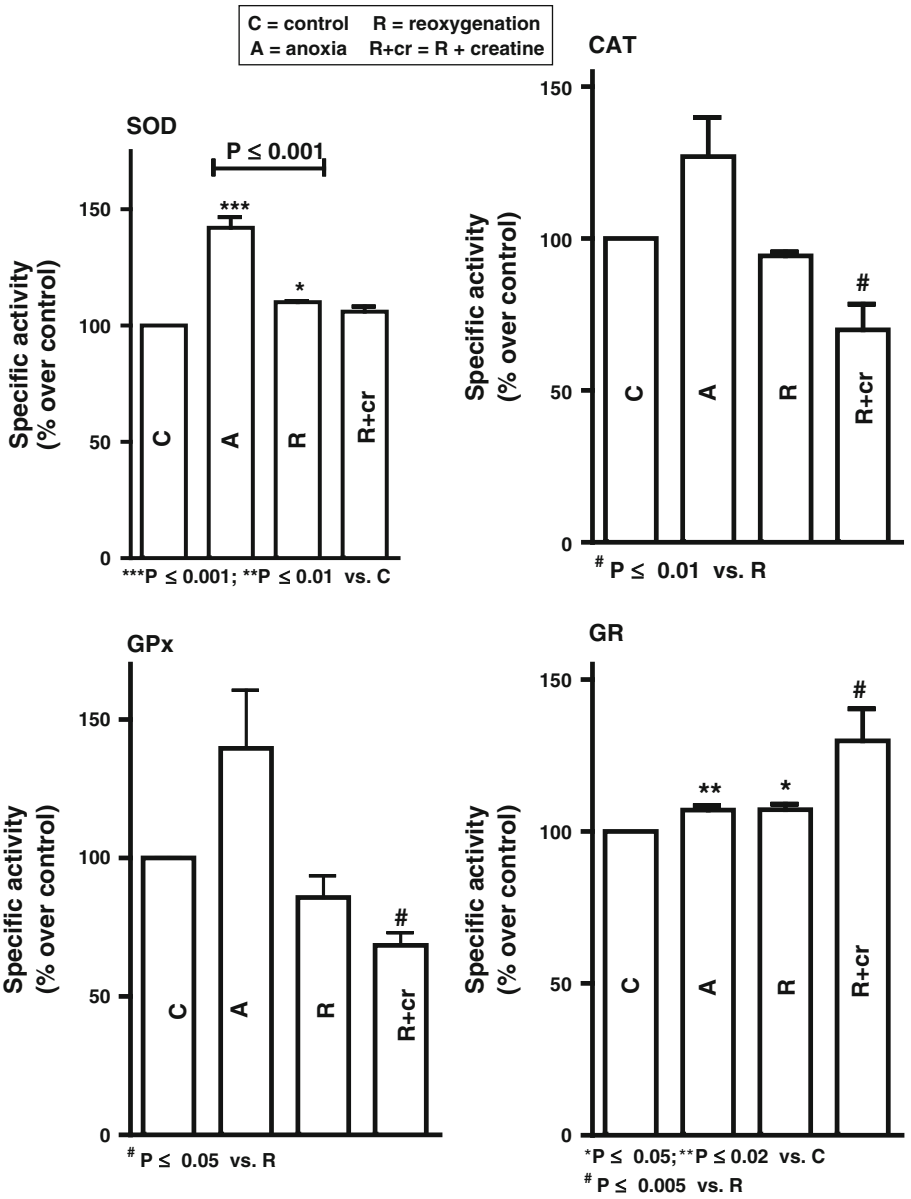


Fig. 3 Effects of anoxia/reoxygenation and 10 mM creatine on antioxidant enzyme activities of ileal mucosal homogenate. Values are means ± S.E. Number of experiments = 5 with duplicate estimation

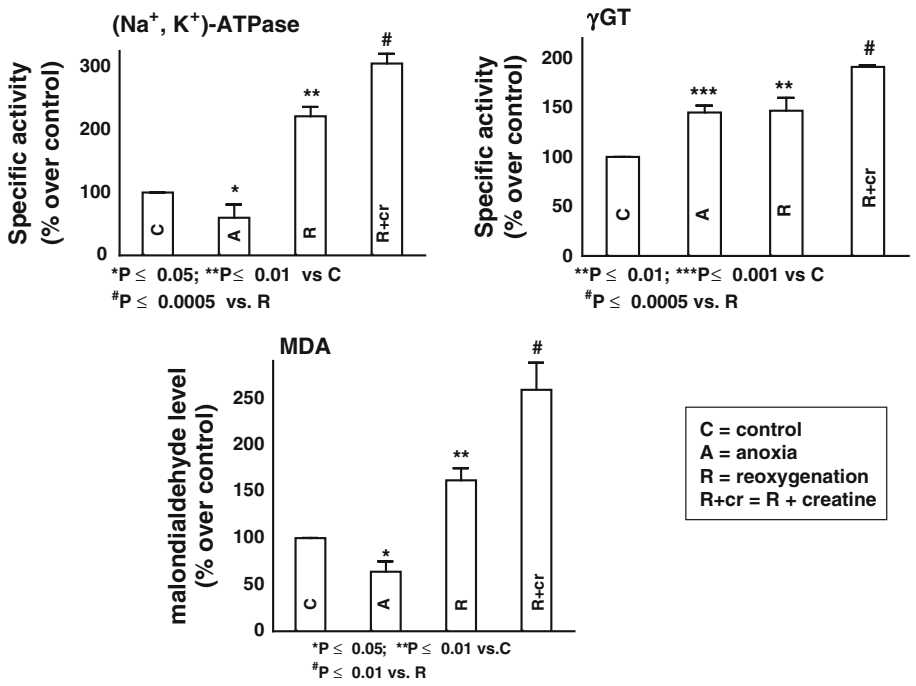
cytosolic enzyme lactate dehydrogenase from both jejunal and ileal mucosa. In jejunum, in anoxia/reoxygenation condition carried out in the absence and in the presence of creatine, LDH mucosal release values (U g wet weight tissue^{-1} 60 min^{-1}) were, respectively, 161.7 ± 10.4 and 117.7 ± 16.7. The correspondent values measured in ileum were 131.7 ± 19.3 and 120.0 ± 16.5 ($n = 4$ in all conditions). No significant differences are evident. Thus, creatine effect does not seem associated with sign of mucosa necrosis.

Effects of reoxygenation and creatine on HSP70 expression in ileal mucosal homogenate

As creatine pretreatment reduces lipid peroxidation during reoxygenation in the ileal tract of small intestine (Fig. 4), we explored whether this effect might be mediated by overexpression of HSP70 induced by creatine. Western blot analysis shows that reoxygenation per se increases HSP70 protein expression and the presence of creatine induces a further significant enhancement (Fig. 5).

Discussion

The intestine is exposed on a daily basis to high levels of oxidative stress and requires strong antioxidants, which may preserve important endocrine, metabolic, immunological and absorptive functions [34]. Thus, it would be important to identify compounds and/or nutrients capable of counteracting the detrimental effect of oxidative stress



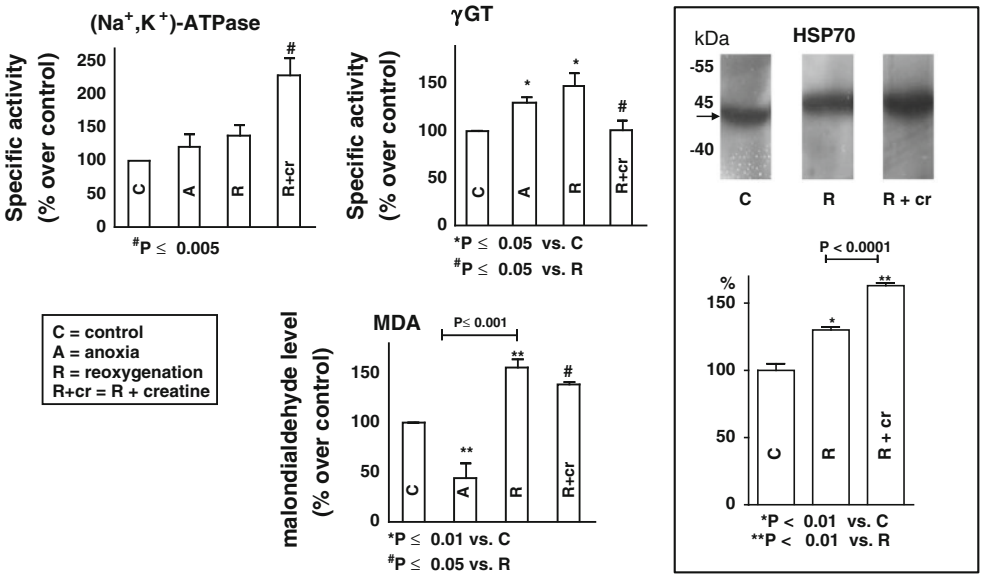


Fig. 4 Effects of anoxia/reoxygenation and 10 mM creatine on γ-GT and (Na^+, K^+)-ATPase activities and MDA production in jejunal mucosal homogenate. Values are means ± S.E. Number of experiments = 5 with duplicate estimation

Fig. 5 Effects of anoxia/reoxygenation and 10 mM creatine on γ-GT and (Na^+, K^+)-ATPase activities, MDA production and HSP70 expression in ileal mucosal homogenate. Values are means ± S.E. Number of experiments = 5 with duplicate estimation. Panel: Western blot analysis for HSP70 was performed on ileum total proteins and was carried out on 4 experiments. Densitometric analysis of the bands reveals significant statistical differences in the intensity of the signals

that arises from dietary oxidized lipids, circulatory oxidized lipoproteins, luminal flora or inflammatory bowel diseases [34, 35].

Creatine is known to exert multiple effects on living cells [16]; since it represents integral part of the human diet, it is highly desirable to understand deeply its biological functions and modes of activity. Aim of this study was to investigate whether an increased availability of creatine could ameliorate the antioxidant response of intestinal cells, rendering them more resistant if undergoing to oxidative stress, in view to lend support to its use in diseases situations.

Intestinal ischemia is a serious state caused by diseases such as acute mesenteric emboli or thrombi, strangulated hernias, severe burns, or traumatic or septic shock. It has been shown in many studies that supplementation of free

radical scavengers is helpful in reducing intestinal I/R-induced tissue damage [33], since oxygen-derived free radicals play a major role in the pathophysiological mechanism of ischemia–reperfusion injury of the intestine [19]. Thus, a further aim of this work was to investigate whether an acute creatine treatment could limit the oxidative injury of the small intestine induced by anoxia and subsequent reoxygenation in rats and whether the protection effects are associated with enhancement of antioxidant capacities.

Major variations in mucosal antioxidant profiles among the different parts of the digestive tract are reported [36]; thus, we have considered two different tracts of small intestine, jejunum and ileum. The effects of creatine differ between the two intestinal tracts, as shown in Tables 1 and 2 and in Fig. 1: as a matter of fact, in jejunum, creatine elicites a significant increase in (Na^+, K^+)-ATPase activity and HSP70 expression, whereas in ileum, (Na^+, K^+)-ATPase and γ-GT are stimulated. Even if a significant stimulation of antioxidant enzyme activities is not apparent, creatine effect appears to be beneficial in both intestinal tracts. In fact, (Na^+, K^+)-ATPase is an essential enzyme involved in transport function of the enterocyte, γ-GT is a key enzymatic activity for the maintenance of GSH homeostasis, and thus for the preservation of antioxidant capacity, while HSP70 is an endogenous factor for protecting cell and tissue under various pathological conditions [31, 32].

Ischemia occurs when local tissue demand for energy substrates is not met by O_2 supply, with hypoxia being an important component of an ischemic insult. Blockage of the oxygen supply causes a decrease in intracellular ATP reserves and a disturbance of cell homeostasis with ROS production [37, 38]. During reperfusion after the resumption of oxygen supply, ROS increase and cause intestinal damage [18, 19, 33]. We used an in vitro model that subjects small intestinal tracts either to anoxia or to transient anoxia followed by reoxygenation. It is known that anoxia gives rise to several compensatory mechanisms acting alone or in concert [39]; the increased SOD, GPx and GR activities observed in jejunum in anoxic conditions (Fig. 2) suggests the involvement of superoxide and hydrogen peroxide in this process, confirming literature data reported for different cell types [18, 19]. Reoxygenation does not evoke further increase in antioxidant activities. In agreement with other studies [33], CAT activity is significantly lowered with respect to the control in all the conditions tested. The decrease in CAT activity could be attributed to the increase in ROS, since CAT scavenged excess free radicals via enzymatic and chemical mechanisms, which resulted in depletion of itself [40]; moreover, increased O_2 may allosterically inactivate CAT enzyme, decreasing its activity [41]. For what concerns cellular antioxidant status, ileal response to anoxia/reoxygenation (Fig. 3) differs to some extent to the jejunal one and seems to be somehow less intense. Interestingly, in both intestinal tracts, a significant increase in γ-GT is apparent in anoxic conditions (Figs. 4 and 5). Increased expression of γ-GT in many cell types is elicited by factors such as inflammation and oxidative stress, which play a role in the etiology of several diseases. It has been proposed that stress-induced upregulation of γ-GT activity is a cellular compensatory mechanism protecting proteins from irreversible oxidative damage [42]. No further increase in γ-GT activity is evident in reoxygenation conditions.

One of the deleterious consequences of oxidative damage is lipid peroxidation, which produces structural and functional damage to membranes as well as several secondary products, among which MDA. MDA production evidenced in Figs. 4 and 5 suggests that antioxidant responses of both jejunum and ileum are effective to avoid tissue injury in anoxic conditions but not following reoxygenation. These results differ from literature data reported for the whole gastrointestinal tract [18, 19] in which tissue damage was already evident after anoxia; however, a similar result was obtained in ileum [43].

As the marker of lipid peroxidation was increased only in reoxygenation, we investigated the possible protective effect of creatine under this condition. In short, the presence of creatine seems to stimulate (Na^+, K^+)-ATPase activity both in jejunum and in ileum (Figs. 4 and 5); this effect could be linked to the role played by creatine in the storage and transmission of phosphate-bound energy [1, 44]. In jejunum, creatine stimulates all the antioxidant activities tested, including γ-GT (Figs. 2, 4), while in ileum, only GR is upregulated (Fig. 3). These findings would indicate that the protective action of creatine is more marked in jejunum; however, unexpectedly, in jejunum, lipid peroxidation level is enhanced by creatine treatment, while in ileum, it is significantly reduced (Figs. 4 and 5). Thus, in jejunum, creatine appears to have both antioxidant and prooxidant activities, as reported for other compounds such as polyphenols and vitamin A [41, 45]. It is worth underlining that the prooxidant effect of creatine is apparent only under reoxygenation conditions and is not associated with sign of mucosa necrosis.

In ileum, creatine effect on cell antioxidant apparatus does not seem sufficiently high to explain the reduction in lipid peroxidation observed in reoxygenation. Results reported in Fig. 5 give evidence for an overexpression of HSP70 in this experimental condition, thus suggesting a role of these proteins in the creatine mode of action in ileum.

To sum up, the two intestinal tracts examined seem to behave differently to the imposed experimental conditions; in particular, a greater stimulation of antioxidant enzyme

activities in response to anoxia is evident in jejunum compared with ileum; the same happens in response to creatine treatment under reoxygenation. However, in both intestinal tracts, the presence of creatine seems to potentiate the defensive response of the tissue, in jejunum by means of cell antioxidant equipment, in ileum by the involvement of HSP70.

Many animal studies have shown that antioxidant agents are useful in protection against I/R-induced gastrointestinal injury [19]. Our data might then concur to lend support to creatine use in these situations. Due to the deleterious effects of prooxidant activity, however, it is essential to understand the prooxidant effect evidenced for creatine in jejunum under reoxygenation conditions.

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