Time-dependent alterations in serum NO concentration after oral administration of L-arginine, L-NAME, and allopurinol in intestinal ischemia/reperfusion

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Objective: To study the effect of oral administration of a nitric oxide (NO) donor L-arginine (L-Arg), a NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and an inhibitor of xanthine oxidase, allopurinol (Allo), on serum NO concentration and catalase activity after intestinal ischemia/reperfusion (I/R) in rats.

Methods: Male Wistar rats received *per os* L-Arg (800 mg/kg) or L-NAME (50 mg/kg) or Allo (100 mg/kg) 24 hrs, 12 hrs and 1 hr before underwent 1 hr occlusion of superior mesenteric artery followed by 1 hr of reperfusion (L-Arg(IR1), L-NAME(IR1) and Allo(IR1) respectively) or 1 hr occlusion followed by 8 hrs of reperfusion (L-Arg(IR8), L-NAME(IR8) and Allo(IR8) respectively). There was one group underwent 1 hr occlusion (I), a group underwent 1 hr occlusion followed by 1 hr reperfusion (IR1), a group subjected to 1 hr occlusion followed by 8 hrs of reperfusion (IR8) and a last group that served as control (C). Serum NO concentration and catalase activity were measured.

Results: After 1 hr of reperfusion serum NO concentration was elevated in IR1 and L-Arg(IR1) groups compared with group C but not in L-NAME(IR1) and Allo(IR1) group. Catalase activity was enhanced in L-NAME(IR1) group. Interestingly, serum NO concentration was increased after 8 hrs of reperfusion in all groups (IR8, L-Arg(IR8), L-NAME(IR8) and Allo(IR8)) compared with control while catalase activity did not show significant difference in any group.

Conclusions: The results of the present study show that NO concentration is elevated in serum after intestinal I/R and the elevation sustained after administration of L-Arg but not after administration of L-NAME or Allo after 1 hr reperfusion. However, after 8 hrs of reperfusion NO concentration was increased in all groups studied, focusing attention on its possible important role in a complicated situation such as intestinal I/R that involves intestine and other organs. Serum catalase activity does not seem to be affected by *per os* supplementation of L-Arg or Allo in intestinal I/R.

Keywords: intestine, ischemia-reperfusion, nitric oxide, L-Arginine, N^G-nitro-L-arginine methyl ester, allopurinol

Introduction

Ischemia/reperfusion (I/R) of mesenteric vessels is involved in several clinical-surgical conditions and often becomes life threaten (Schoenberg and Beger 1993; Massberg and Messmer 1998; Oldenburg et al 2004) Discontinuation of nutritive blood flow to the intestine leads to tissue damage, which can be irreversible. During ischemia, but especially during reperfusion, inflammatory factors are generated, cytotoxic substances are released, enzymes and immune cells are implicated in tissue injury. Mechanisms underlying the pathophysiology of I/R are not clearly understood (Takada et al 1998; Cerquiera et al 2005).

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A large number of studies have been focused on nitric oxide (NO) (Moncada et al 1991; Kurose et al 1994; Takada et al 1998; Cerquiera et al 2005), a highly reactive substance with a highly conflicting role. NO exerts beneficial effects as a strong vasodilator and protector of vascular integrity. There are studies showing that NO reduces mucosal injury after I/R (Kubes and Granger 1992) and decreases lung injury caused by intestinal ischemia (Terada et al 1996). On the other hand NO reacts with superoxide anion leading to the formation of peroxinitrite (ONOO-) an oxidative agent capable to cause tissue damage (Takada et al 1998; Cuzzocrea et al 1998).

The aim of the present study is to investigate the effect of oral administration of substances that are related to NO metabolism in serum NO concentration in rats underwent superior mesenteric artery ischemia followed by 1 hr or 8 hrs of reperfusion. A NO donor L-arginine (L-Arg), a NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) and an antioxidant allopurinol (Allo) were studied.

Although there is no direct link between NO metabolism and catalase activity, assessment of an antioxidant enzyme in a process such as ischemia/reperfusion where oxidative stress plays major role in tissue injury, in combination with a possible oxidative role of NO supported by bibliography, is thought to be important (Kacmaz et al 1999; Song et al 2007). Hence, the effect of the above mentioned substances in the antioxidant enzyme catalase under I/R was also given attention.

Methods

Animals

Male Wistar rats (2 months old) weighting 250–350 g participated in the study. Animals were fed a standard rat chow and had free access to tap water. They were housed in groups of two, in cages with wood shaving as bedding in an air conditioned room with 20 ± 2 °C temperature, 55 ± 10% humidity and 12 hr light-dark cycle (light on at 5:30 a.m.). Rats were left to adapt in the laboratory environment for 1 week. These animals had not participated in any other experimental procedure before and a veterinarian was observing their health and general condition during the adaptation period. 24 hrs prior to experimentation animals were fasted but free access to water was allowed.

All protocols were approved by the Institute's Ethics Committee and conducted in compliance with the European Convention on Animal Care.

Experimental protocol

Rats were divided into ten groups of 8 animals each. In group C (control) animals did not receive any substance. In group I animals underwent 1 hr occlusion of superior mesenteric artery while in groups IR1 and IR8 1 hr occlusion was followed by 1 hr and 8 hrs reperfusion respectively. The rest groups received *per os* L-Arg (800 mg/kg) or L-NAME (50 mg/kg) or Allo (100 mg/kg) 24 hrs, 12 hrs and 1 hr in equal doses before underwent 1 hr occlusion of superior mesenteric artery followed by 1 hr reperfusion (L-Arg(IR1), L-NAME(IR1) and Allo(IR1) respectively) or 1 hr occlusion of superior mesenteric artery followed by 8 hrs reperfusion (L-Arg(IR8), L-NAME(IR8) and Allo(IR8) respectively).

Anesthesia was induced by intramuscular injection of xylazine (10 mg/kg) and ketamine (100 mg/kg) and animals were placed on heating pads for maintenance of body temperature at 37 °C. Supplementary half of the initial dose was given intraperitonealy 40 min after the beginning of the surgical operation. Animals in group C subjected to a midline abdominal incision and sacrificed after blood collection from inferior vena cava. In rest groups, superior mesenteric artery was isolated and occluded with an atraumatic microvascular clamp for 1 hr to obtain ischemia. After this period of time clamp was removed and the reperfusion period started with return of mesenteric blood flow in all groups.

Analyses of NO and catalase

Total NO concentration was measured in serum with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Assay Designs, Inc, Ann Arbor, MI). The method is based on Griess reaction and determines the two stable breakdown products of NO, nitrite, and nitrate. Briefly, nitrate is enzymatically converted to nitrite by the enzyme nitrate reductase. Nitrite is measured as an azo dye product of Griess reaction that absorbs light at 540 nm.

Catalase activity was also measured in serum by an ELISA kit (Cayman Chemical, Ann Arbor, MI). The method is based on the production of a purple product via the reaction of formaldehyde with a chromogen. Formaldehyde is formed by catalase action on methanol in the presence of H_2O_2.

Statistical analysis

Statistical analysis was performed using SPSS 10.0 statistical software. Data are presented as mean ± standard error of mean (SEM). Statistical significance was determined by Student's *t*-test for independent samples. p value less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

Serum NO concentration was elevated significantly after 1 hr reperfusion and after 1 hr reperfusion with prior administration

of L-Arg (Figure 1). Ischemia did not increase NO concentration, since in group I, NO levels did not have any statistical difference from group C ($p > 0.05$). However, 1 hr reperfusion followed resulted in significant increase (85.5 ± 15.06 in IR1 group *vs* 33.91 ± 5.71 μmol/L in group C, mean ± SEM, $p < 0.05$). L-Arg maintained serum NO elevation since the animals that received L-Arg prior to I/R had higher levels of NO when compared with control (94.77 ± 15.80 in L-Arg(IR1) group *vs* 33.91 ± 5.71 μmol/L in group C, $p < 0.05$).

NO levels in L-NAME(IR1) group did not have any significant difference from control animals. Probably L-NAME prevented NO elevation, which is caused by reperfusion during the first hr ($p > 0.05$).

In the group of animals received allopourinol, Allo(IR1), serum NO concentration was 34.60 ± 7.35 μmol/L after 1 hr ischemia followed by 1 hr reperfusion and did not have any statistical difference from control group ($p > 0.05$).

Serum NO levels were significantly elevated in all treatment groups compared to control when ischemia was followed by 8 hrs of reperfusion. Hence, NO values were 94.85 ± 15.10 in L-Arg(IR8) group, 104.70 ± 17.10 in L-NAME(IR8) group and 94.38 ± 18.61 μmol/L in Allo(IR8) group ($p < 0.05$ *vs* control).

L-Arg and Allo did not seem to affect catalase activity in serum as it is presented in Table 1. However, its activity was significantly higher in L-NAME(IR1) group (55.98 ± 5.95 *vs* 33.16 ± 2.72 nmol/min/mL in group C, $p > 0.05$; Table 1). After 8 hrs of reperfusion this effect did not exist.

Discussion

The present study was designed to set light on the mechanisms underlying the metabolic processes involved in intestinal I/R related to release of substances into circulation, which may affect the intestine or other organs. Due to the fact that a large number of studies have been focused on the role of NO and antioxidant enzymes in intestinal I/R, the effect of oral administration of L-Arg, L-NAME, and allopurinol on serum NO concentration and catalase activity in intestinal I/R was studied.

L-Arg is the precursor of NO synthesis via the reaction that converts L-Arg to L-citrulline and catalyzed by NO synthase (NOS). NOS exists in various isoforms. Studies have shown that among them, inducible form (iNOS) is thought to be the one leads to the formation of excessive NO that causes tissue injury in intestinal I/R (Gross and Wolin 1995; Naito et al 2002). However, L-Arg administration has been shown to ameliorate serum and pulmonary cytokine response after gut I/R (Fu et al 2005) and to exert protective effects on reperfusion after pancreaticoduodenal transplantation in rats (Yuan et al 2004). L-NAME is a potent competitive NOS inhibitor that restricts NO formation and allopurinol is a competitive inhibitor of xanthine oxidase, an enzyme that is strongly related to I/R injury. Xanthine oxidase is another form of xanthine deydrogenase which normally catalyses the metabolism of

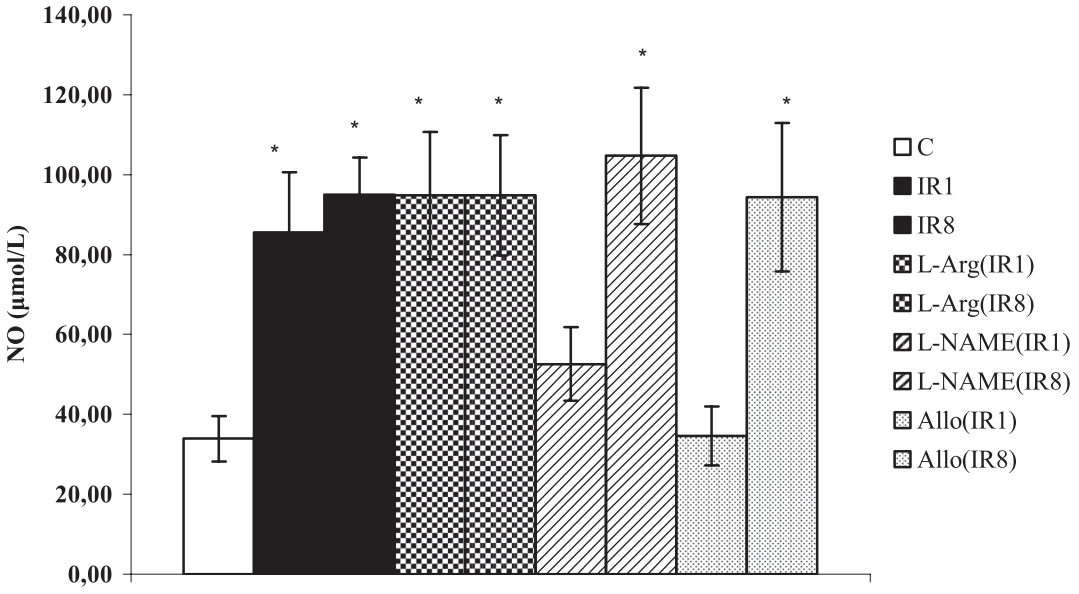

Figure 1 Time-dependent alterations in serum NO concentration after oral administration of L-Arg, L-NAME and Allo in 1 hr ischemia followed by 1 hr (L-Arg(IR1), L-NAME(IR1) and Allo(IR1)) or 8 hrs (L-Arg(IR8), L-NAME(IR8) and Allo(IR8)) of reperfusion. After 8 hrs of reperfusion all treated groups have elevated serum NO concentration. *$p < 0.05$ compared to group C.

Table 1 Effect of L-Arg, L-NAME and Allo on catalase activity after I/R. Values are expressed as means ± SEM

Group	Catalase (nmol/min/mL)
C	33.16 ± 2.72
I	41.73 ± 6.03
IR1	65.61 ± 17.22
L-Arg(IR1)	57.79 ± 17.52
L-NAME(IR1)	55.98 ± 5.95[a]
Allo(IR1)	37.78 ± 2.28
IR8	151.58 ± 65.69
L-Arg(IR8)	150.16 ± 60.92
L-NAME(IR8)	168.06 ± 63.29
Allo(IR8)	53.81 ± 13.15

Notes: [a]$p < 0.05$ compared to group C.

xanthine to uric acid with NAD^+ as electron acceptor. During ischemia xanthine dehydrogenase is converted to xanthine oxidase which catalyses the reaction of xanthine to hypoxanthine that is subsequently accumulated in intestinal tissue due to ischemia. After reoxygenation hypoxanthine is degradated to uric acid using molecular oxygen as electron acceptor and leads to the formation of free radicals (Schoenberg and Beger 1993). Allopurinol decreases the rate of formation of free radicals and prevents tissue injury after reperfusion (Parks and Granger 1983; Schoenberg and Beger 1993). Additionally, xanthine oxidase induces retention of neutrophils in the lung after intestinal I/R (Terada et al 1996).

The results of the present study revealed that serum NO concentration was raised after I/R. This finding has also been demonstrated by other researchers (Xia et al 2001). It is important to note that NO concentration was not affected by ischemia *per se* but only after reperfusion in which the major alterations in tissue metabolism are attributed. This rise in serum NO may reflect a protective mechanism for secondarily injured organs. It has been suggested that even 30 minutes of intestinal I/R can initiate pulmonary inflammation and that endogenous NO limits lung injury after intestinal I/R in rats (Terada et al 1996). Accordingly, L-Arg administration maintained elevated serum NO concentration after I/R and may contribute to endogenous NO protection. However, L-Arg did not result in an additional rise of NO showing that this enhancement of concentration maybe closed under limits. Interestingly, NO concentration after L-Arg administration remained almost constant during 8 hrs of reperfusion.

L-NAME and Allo did not have any effect on serum NO levels compared with control after 1 hr reperfusion. L-NAME as a competitive inhibitor of NOS probably restricted NO production that observed in I/R. It is important to note that L-NAME administration resulted in high mortality of the animals during reperfusion. In contrary, animals received L-Arg were in a very good general condition post-operatively and this observation enhances the possibility of a beneficial role of NO.

Allopurinol is a substance known for protection from tissue damage and its existence may prevented serum NO increment (or made it not necessary). This hypothesis is supported by the elevation of serum NO concentration after 8 hrs of reperfusion when these substances would have been catabolized and NO production follows that observed in groups of IR and L-Arg. Additionally, animals post-operative condition was very good after Allo administration. These time-dependent alterations in NO concentration may reflect a trend of the body to produce this substance via an endogenous protective mechanism which prevents inflammation in other implicated to intestinal I/R, organs.

On the other hand, L-NAME gave rise to catalase activity in serum after 1 hr reperfusion that did not seem to be affected by ischemia, I/R and L-Arg or Allo administration. This finding is difficult to be explained. Since the antioxidant activity is higher than control group our thought is led to the existence of an equilibrium between NO generated free radicals and antioxidants in normal conditions which favors antioxidants when L-NAME is administered. Oral administration of L-NAME has been shown to increase catalase activity also in other situations (Mara et al 2007). More studies are needed in order to support the above-mentioned hypothesis.

Since reperfusion causes large metabolic alterations and NO concentration increased after I/R it would be wise to consider the effect of this release of NO in the circulation in other organs where it could be beneficial. On the other hand the increase of catalase activity by L-NAME reflects the complexity of NO metabolism and its different role regarding different tissues.

More studies are needed for the elucidation of the mechanisms underlying NO metabolic responses after intestinal I/R.

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