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Original Article

Effect of Ornithine on the Ileal Histology, Nitric Oxide Production and Lipid Peroxidation in LPS-Induced Endotoxemia

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Effect of ornithine which is known to inhibit L-arginine uptake via cationic amino acid transport system has been tested, and compared to aminoguanidine, an iNOS inhibitor in lypopolysaccharide (LPS)-induced endotoxemia in rats. Serum nitrite/nitrate and malondialdehyde (MDA) level have been measured, and ileal histology has also been examined. Endotoxin increased serum nitrite/nitrate and MDA levels from 15.7 ± 2.4 μmol/ml and 2.1 ± 0.2 nmol/ml to 23.1 ± 1.0 μmol/ml and 5.2 ± 0.3 nmol/ml (both $P < 0.05$), respectively. In addition, LPS caused ileal degenaration. L-ornithine (500 mg/kg) did not improve septic manifestations, *i.e.*, serum nitrite/nitrate and MDA levels did not differ from those in endotoxemia. Neither does it have an improving action on ileal histology. However, higher dose of L-ornithine (2,500 mg/kg) lowered the increased level of nitrite/nitrate and MDA by LPS. Moreover, it restored ileal histology from grade 3 (median) to 0 (median) ($P < 0.05$). On the other hand, aminoguanidine (100 mg/kg) normalized serum nitrite/nitrate and MDA levels but not ileal histology in endotoxemic rats. In conclusion, high dose of L-ornithine could improve endotoxemic parameters in LPS-treated rats.

Key words: LPS, ornithine, aminoguanidine, endotoxemia, lipid peroxidation

Bacterial lipopolysaccharide (LPS) or endotoxin is an initiator of the septic syndrome that is recognised as a major cause of multiorgan dysfunction syndrome [1]. Endotoxin triggers the release of numerous mediators including nitric oxide and reactive oxygen species (ROS) which activate a variety of pathological mechanisms that culminate in tissue dysfunction and organ failure [2].

It has been demonstrated that endotoxemia increases circulating level of nitrite/nitrate (NO_2^-, NO_3^-), stable products of NO in various animals as well as human sepsis [3, 4]. Massive NO production which probably accounts for tissue damage in endotoxemia results from the induction of inducible nitric oxide synthase (iNOS) [5]. Therefore, inhibition of NO as well as ROS production seems to be vital in the treatment of septic syndrome.

NO is synthesized from a semiessential aminoacid, L-arginine and, its analogues have been used as inhibitors of NOS [6]. L-arginine is mainly transported into endothelial cells via cationic aminoacid transport system (y^+). L-lysine and L-ornithine are known to inhibit this system [7]. Although effect of L-lysine has been tested in ovine endotoxemia [8], there is no report with regard to ornithine, another chemically-related amino acid in LPS-induced endotoxemia in rats. For that reason, we

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have aimed to investigate if ornithine has any beneficial effects in rat endotoxemia by evaluating levels of serum nitrite/nitrate and malodialdehyde (MDA), indicators of NO and lipid peroxidation respectively. Further, we have examined any effects of the aminoacid on ileal histology.

Materials and Methods

The experiments in this study were conducted in adherence to the rules of the local ethic commitee. Female Wistar rats weighing 170–200 g were housed at constant temperature with 12/12 h periods of light and dark exposure. The animals had free access to standard rat chow and water *ad libitum* during acclimation period of at least 5 days. After fasting over night in the morning at 10 o'clock the rats were randomly divided into 8 groups.

Group 1: Served as control, received intraperitoneal saline (1 ml/200 g, n = 6);
Group 2: Endotoxin (*Escherichia coli* lipopolysaccharide, 055:B5, 10 mg/kg, i.p., Sigma, St. Louis, MO, USA, n = 5),
Group 3: I.p. ornithine (500 mg/kg, Sigma, St. Louis, MO, USA, n = 4),
Group 4: I.p. ornithine (2,500 mg/kg, n = 5),
Group 5: I.p. aminoguanidine (100 mg/kg, n = 5)
Group 6: I.p. ornithine (500 mg/kg) 10 min before endotoxin injection (n = 6).
Group 7: I.p. ornithine (2,500 mg/kg) 10 min before endotoxin injection (n = 6).
Group 8: I.p. aminoguanidine (100 mg/kg) 10 min before endotoxin injection (n = 7).

Six hours later, rats anaesthetized with intramuscular ketamine (50 mg/kg) and the blood was taken by cardiac puncture for nitrite/nitrate levels. In order to evaluate the endotoxin-associated ileal injury, tissue samples were harvested through a midline incision, and fixed in 10% formaldehyde.

Detection of Serum Nitrite/Nitrate Level. In biological fluids NO is very rapidly deactivated by oxidation to nitrite and nitrate. After collecting the blood samples by cardiac puncture, we detected NO via nitrite/nitrate. Nitrate was reduced to nitrite by NADPH in the presence of nitrate reductase and the formed nitrite was put to react with sulphanilamide and N-(1-naphtyl)-ethylenediamine to give a red-violet diazo dye. The diazo dye measured on the basis of its absorbance in the visible range at 550 nm.

Lipid Peroxidation Assay. The levels of serum lipid peroxidation products as thiobarbituric acid (TBA)-malondialdehyde (MDA) adducts were measured spectrophotometrically by the method described by Yagi [9]. The final results were expressed as nmol of MDA formed per mililiter of serum.

Histological Examination of ileal segments. The ileal specimens were fixed in 10% formaldehyde. Hematoxylin and eosin-stained slides were prepared by using standard methods. Intestinal mucosal damage was graded on a 6-tiered scale as defined by Chiu *et al.* [10] in a minimum of 20 seperate locations on each sections. Briefly, mucosal damage was graded from 0 to 5 according to the following criteria:

Grade 0: Normal mucosal villi
Grade 1: Development of subepithelial space
Grade 2: Extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria
Grade 3: Massive epithelial lifting down the side of the villi
Grade 4: Denuded villi with lamina propria and dilated capillaries exposed
Grade 5: Digestion and disintegration of lamina propria; hemorrhage and ulceration

Statistical Analysis. Values are given as mean ± S.E.M., or median and quartiles. Statistical differences for serum nitrite/nitrate and MDA values were evaluated using one way of ANOVA followed Bonferroni post hoc test. Comparison for intestinal injury scores was analyzed using Kruskall-Wallis variance analysis followed by Dunn test. P values less than 0.05 were considered significant.

Results

Serum Nitrite/Nitrate Levels. No animals died during the experimentation. As shown in Table 1, serum nitrite/nitrate level was found to be increased in endotoxemic group (15.72 ± 2.43 μmol/l *vs.* 23.10 ± 1.0 μmol/l, $P < 0.05$). While treatment of ornithine (500 mg/kg) failed to prevent the ability of LPS to elevate serum nitrite/nitrate level, higher dose of ornithine (2,500 mg/kg) markedly decreased that level ($P < 0.05$). Furthermore, aminoguanidine (100 mg/kg) significantly lowered nitrite/nitrate level ($P < 0.05$).

Lipid Peroxidation. In LPS-injected group the level of serum malondialdehyde (MDA) was 5.20 ± 0.30 nmol/ml, whereas it was 2.12 ± 0.21 nmol/ml in

Table I Serum nitrite/nitrate and malondialdehyde (MDA) levels in all groups.

Group	Nitrite + Nitrate (mmol/ml)	MDA (nmol/ml)
Control (n = 6)	15.70 ± 2.40	2.12 ± 0.21
LPS (n = 8)	23.10 ± 1.04*	5.20 ± 0.30*
Ornithine (500 mg/kg, n = 4)	8.67 ± 2.62	1.24 ± 0.11
Ornithine (2,500 mg/kg, n = 5)	8.10 ± 0.43	1.16 ± 0.05
Aminoguanidine (100 mg/kg, n = 5)	14.20 ± 0.37	1.04 ± 0.24
LPS + Ornithine (500 mg/kg, n = 6)	21.00 ± 2.90	4.10 ± 0.73
LPS + Ornithine (2,500 mg/kg, n = 6)	17.50 ± 0.45**	3.05 ± 0.13**
LPS + Aminoguanidine (100 mg/kg, n = 7)	13.10 ± 0.49**	1.50 ± 0.13**

Data are expressed as mean ± S.E.M. *, Different from control; **, Different from LPS-treated group. Statistical differences were evaluated using one way of ANOVA followed Bonferroni post hoc test.

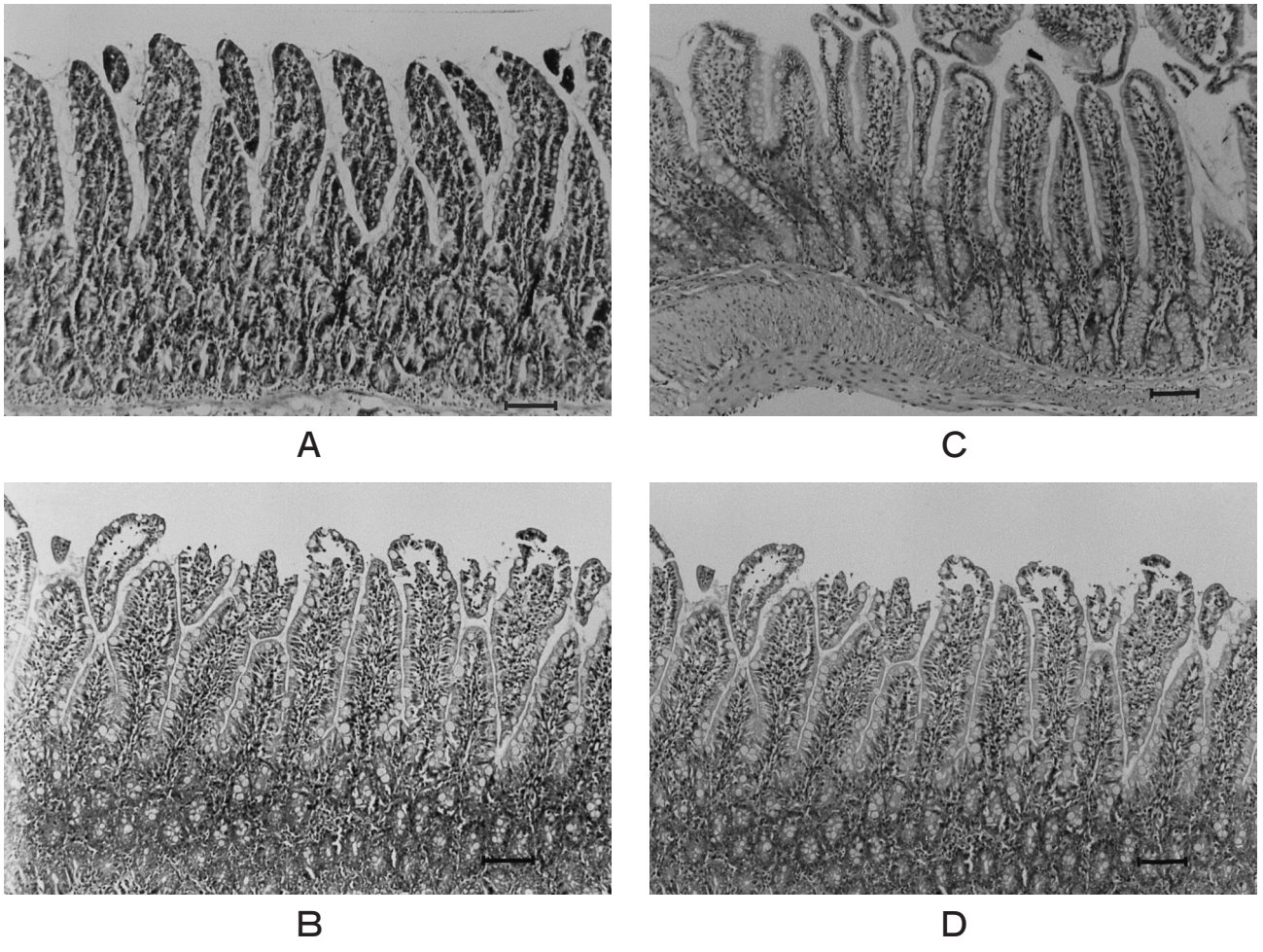

Fig. 1 Photomicrographs of small intestine segments (H & E × 200).
A, Control group showing normal histology; **B**, LPS only group showing massive epithelial lifting down the sides of villi with a few denuded tips; **C**, Ornithine (2,500 mg/kg) reduced the LPS induced intestinal injury ($P < 0.05$); **D**, Aminoguanidine (100 mg/kg) did not improve ileal damage by LPS administration. Bars indicate 100 μm

control which is statistically different ($P < 0.001$). In ornithine alone injected groups, the levels of MDA were 1.24 ± 0.11 nmol/ml and 1.16 ± 0.05 at the doses of 500 mg/kg and 2,500 mg/kg, respectively. In the serum from the rats given LPS and 500 mg/kg ornithine MDA levels were 4.10 ± 0.73 nmol/ml which was not significantly different from LPS-injected group. However, at the dose of 2,500 mg/kg, ornithine significantly decreased MDA level from 5.20 ± 0.30 nmol/ml to 3.10 ± 0.13 ($P < 0.01$, Table 1). Similarly aminoguanidine lowered both nitrite/nitrate and MDA levels in endotoxemic rats (Table 1).

Light Microscopic Findings. Small intestine of the rats was assessed for tissue damage by histologic examination. As shown in Fig. 1B, endotoxemia caused significant tissue damage. These changes varied from denuded villi with exposed dilated capillaries to significant architectural distortion, lamina propria disintegration, ulceration and hemorrhage. The histopathologic scores of control and ornithine groups (500 mg/kg and 2,500 mg/kg) were significantly smaller than LPS group (Fig. 2). LPS plus ornithine (500 mg/kg) did not reduce the LPS induced intestinal injury. There was no statistical significant difference between the scores of LPS and LPS plus ornithine (500 mg/kg) group (Fig. 2). However, 2,500 mg/kg ornithine normalized the intestinal damage by LPS (Fig. 1C). The Chui scores of this group were shown in Fig. 2. However, aminoguanidine (100 mg/kg) did not prevent LPS-induced ileal injury (Fig. 1D, Fig. 2).

Discussion

Selective inhibition of iNOS which is induced by bacterial LPS is of value in the treatment of septic or endotoxemic states as over-produced NO has deleterious effects on tissue histology [11, 12]. This can be achived by some spesific iNOS inhibitors such as N-(1-iminoethyl)-L-lysine (L-NIL) and 1,400 W or by some nonspesific inhibitors of iNOS namely aminoguanidine [13–15]. However, it has been reported that large doses of non-selective NOS inhibitors may increase mortality, and the mechanism underlying this effect is not clear but might include impaired tissue perfusion due to excessive vasoconstriction, excessive suppression of cardiac contractility or inhibition of 'physiological' NO, which might be exerting cytoprotective effects [11, 12].

On the other hand, uptake of L-arginine has been proposed to be rate limiting under certain conditions for NO production [16]. These conditions most probably involve septic and endotoxemic states since iNOS needs further L-arginine after consuming up the intracellular store of the aminoacid to generate NO with a massive amount. Inhibition of the cationic aminoacid transport

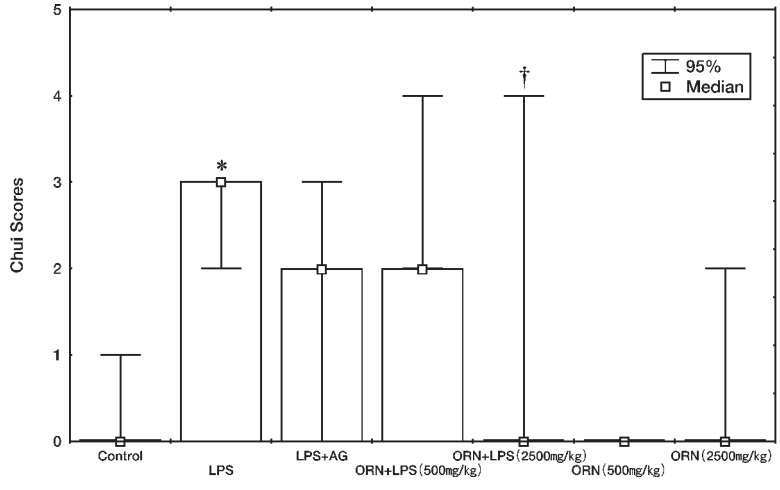

Fig. 2 Histopathologic scores of the bowel specimens obtained from the study groups. Treatment of L-ornithine (2,500 mg/kg) but not aminoguanidine (100 mg/kg) decreased ileal injury score by LPS administration. *, the difference from control; †, from LPS-administrated group. Comparison for intestinal injury scores was analyzed using Kruskall-Wallis variance analysis followed by Dunn test. Results are presented median and quartiles. The value of 95% indicates the quartiles and the rest is 5%. *P* values less than 0.05 were considered significant.

system with other chemically-related aminoacids such as L-lysine or L-ornithine may take an advantage by restricting the extra supply of L-arginine in endotoxemic condition but not the physiological formation of NO by the constitutive NOSs because intracellular L-arginine store is sufficient for those enzyme-catalysed NO production. Indeed, it has been demonstrated that L-arginine uptake is inhibited by L-lysine, L-homoarginine and L-ornithine [17, 18]. It has been suggested that constitutive NO formation be unaffected in sepsis or related states as it is physiologically necessary for the regulation hemodynamic parameters [11, 19]. One attempt has been made in ovine endotoxemia with L-lysine. However, the amino acid failed to restore the refractory hypotension but did L-NAME, a non-specific NOS inhibitor [8]. Neither did L-lysine change serum nitrite/nitrate level. Conversely, in rat endotoxemic model L-lysine administration caused the inhibition of NO production by iNOS but not by constitutive NOS (cNOS) [20]. In the present study, however, we have tried another inhibitor aminoacid, ornithine for the y^+ system. Smaller dose of ornithine had no improving effects on either ileal histology or serum nitrite/nitrate level as well as lipid peroxidation in endotoxemic condition. One reason for the failure seems to be due to the insufficient amount of L-ornithine given. Accordingly, we examined higher dose (2,500 mg/kg), and it restored endotoxin-induced ileal damage. Likewise, the dose dramatically lowered both nitrite/nitrate and MDA levels, supporting the restorative effects of the aminoacid. On the other hand L-ornithine significantly decreased LPS-induced nitrite accumulation in murine bone marrow derived macrophages [21], confirming the results of our study. Apart from the relationship of L-ornithine with L-arginine: NO pathway, the aminoacid is also a substrate for poliamine synthesis. It is not known whether poliamine synthesis is increased with ornithine treatment, and possible increased level of poliamines have any effects on the parameters in this study. It has been reported that NO can inhibit ornithine decarboxylase which is the initial and rate limiting step in poliamine synthesis that is necessary for cell growth in mammals [22].

On the other hand, marginally-spesific iNOS inhibitor, aminoguanidine did not improve ileal damage, although it dramatically diminished nitrite/nitrate overproduction and MDA levels in the study. This may reflect that restorative effect of ornithine might not be parellel with the decreased amount of nitrite/nitrate and MDA, or it has improving effect via different mode of action, or aminoguanidine itself has deleterious effect on the ileal histology although it substantially lowered both MDA and nitrite/nitrate levels.

In conclusion, L-arginine uptake which is the rate limiting for NO biosynthesis can be inhibited by high dose of L-ornithine. Therefore, it seems to be a beneficial agent which can reduce noxious NO formation that could mediate tissue damage in the rat endotoxemia.

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