

Increase in Rat Plasma Antioxidant Activity after *E. coli* Lipopolysaccharide Administration

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It is well recognized that the sensitivity of animals to lipopolysaccharide (LPS) endotoxin varies tremendously. And, it has been recently observed that Sprague-Dawley rats dramatically increase the activity of hepatic endogenous antioxidative enzyme systems after LPS administration. This finding suggests that the relative resistance of rats to LPS may be related to a concomitant increase in the activities of the hepatic antioxidant systems. This study was designed to examine if the above reported hepatic change in rats given LPS could be observed at the systemic level. Male Sprague-Dawley or Wistar rats, weighing 250-350 g, were given increasing doses (10-100 mg/kg) of LPS i.p. under 1.0% isoflurane anesthesia. Antioxidant capacity (AOC), blood gas analysis, and the cardiovascular parameters of the arterial blood of animals were determined over a 4 hour period following LPS administration. In addition, we studied the effect of pretreatment with the non-specific nitric oxide synthase inhibitor, L-N^G-Nitroarginine methyl ester hydrochloride (L-NAME), given 50 mg/kg s.c. one and 24 hours before the administration of 20 mg/kg LPS i.p. in Sprague-Dawley rats. Rats given sufficiently high doses of *E. coli* LPS to produce behavioral effects also showed increased plasma AOCs in the early period after the administration of LPS. Similar changes were noted in Sprague-Dawley and Wistar rat strains, but at different doses that reflect their differential sensitivities to the LPS induced inflammatory response. Also, the resistance of the Sprague-Dawley strain of rats to LPS was not altered by the prior administration of L-NAME, nor was the plasma AOC altered. In conclusion, our study suggests that the rat strains are relatively resistant to develop the toxic signs of LPS in the early period after the administration of LPS, especially in

Sprague-Dawley rats. Moreover, endotoxin-induced increases in plasma AOC may contribute to the rats' resistance to LPS intoxication.

Key Words: *E. coli* endotoxin, antioxidant capacity, nitric oxide synthase inhibition, blood pressure, arterial blood gas

INTRODUCTION

It is well known that the sensitivity of animals to lipopolysaccharide (LPS) endotoxin varies tremendously.^{1,2} Thus, rats and mice tend to be resistant to its inflammatory and cardiovascular effects and require doses in the mg/kg range, whereas rabbits and humans are sensitive to μ g/kg quantities.

Shaul et al recently observed that Sprague-Dawley rats dramatically increase the activity of hepatic endogenous antioxidative enzyme systems after LPS administration, whereas less profound or even opposite changes were observed in the livers of rabbits given LPS.² In addition, the above study also reported a decrease in hepatic malondialdehyde levels in rats after LPS treatment. These findings suggest that the relative resistance of rats to LPS may be related to a concomitant increase of activity in the hepatic antioxidant systems.

The present study was designed to examine if the above reported hepatic changes in rats given LPS could be observed at the systemic level. Thus, the cardiovascular functional parameters and the antioxidant capacity (AOC) of the arterial blood of

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rats given increasing doses of LPS were determined.

Nitric oxide (NO) is known to play a key modulatory role in the development of LPS or sepsis-induced shock.³ For this reason, the initial vasodilation reported in some species can be blocked by the inhibition of endothelial nitric oxide synthase (NOS).⁴ NO administration has also been reported to inhibit detrimental LPS effects *in vitro*⁵ and *in vivo*.⁶ Since rats produce a large amount of nitrate/nitrite relative to other species more sensitive to LPS-induced toxicity,³ we explored whether reduced NO availability would provoke a hypotensive or a greater metabolic response in the Sprague-Dawley rats after LPS administration.

METHODS AND MATERIALS

Male Sprague-Dawley or Wistar rats, weighing 250-350 g, were obtained from Charles River, and used in a protocol approved by the Institutional Animal Rights Review Board of the University of North Carolina. LPS from *Escherichia coli* (0111: B4 from Sigma Chemical Co., St. Louis, USA) was diluted in 0.9% saline, sonicated, and injected i.p. at 2 mL/kg. Control animals were run concurrently, and received saline alone (2 mL/kg) by the same route.

L-N^G-nitroarginine methyl ester hydrochloride (L-NAME), a nonspecific NO synthase inhibitor, was purchased from the Sigma Chemical Co., St. Louis, USA, and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chromon-2-carboxylic acid, Aldrich Chemical, Milwaukee, USA), a water-soluble analog of vitamin E, was used to standardize AOC.

Animal Protocol

In order to obtain arterial blood to measure antioxidant activity, blood gas analysis, and cardiovascular parameter, rats were lightly anesthetized with diethyl ether, and a 16 G IV cannula was inserted through the mouth into the trachea (verified by a small incision in the strap muscles) and a femoral artery was cannulated with PE50 tapered polyethylene tubing. The neck and groin incisions were each swabbed with 0.1 ml of 2%

lidocaine. The spontaneously breathing rats were then given 1.0% isoflurane in air via a T-piece circuit attached to the tracheal cannula.

After 20 minutes of equilibration, and immediately before the administration of increasing doses of 10 - 100 mg/kg LPS or saline (control) i.p., arterial blood was removed to measure blood gases and pH (0.3 mL of blood aspirated into heparinized syringes) and plasma AOC (0.5 mL of blood into syringes containing 2.0 mL heparinized saline, 10 units heparin/mL of saline, chilled on ice). Rats were then given 0.75 mL/hr of heparinized saline (10 units heparin/mL of saline) via the femoral catheter and 0.8 mL of replacement heparinized saline slowly through the femoral catheter over 1 - 2 minutes after each blood withdrawal. Similar volumes were removed (and replaced) 30, 120, 180, and 240 minutes after the administration of LPS or saline. Rectal temperature was maintained at 34 - 36°C using a Yellow Springs rectal thermometer and a heating pad with cloth cover.

In order to reduce NO synthesis from all 3 nitric oxide synthase (NOS) isoenzymes, six Sprague-Dawley rats were pretreated with the non-specific NOS inhibitor, L-NAME, at 50 mg/kg in a volume of 2 mL/kg saline s.c., one and 24 hours before LPS administration.⁷ Another six control Sprague-Dawley rats were given saline alone, and the same methods as mentioned above were used to determine antioxidant activity, blood gases, and cardiovascular parameters after 20 mg/kg of LPS was administered i.p..

Sample Handling

Immediately after withdrawal, arterial blood gases were analyzed on a Radiometer (model ABL5) blood gas machine standardized daily for pH, PaCO₂, and PaO₂. Diluted blood was rapidly centrifuged (2,000 × g for 10 minutes) to remove cells, and the diluted plasma layer was removed and frozen at -40 to -80°C for less than one week before analysis for AOC by a modification of the method of Visioli and Galli⁸ and for protein by the Pierce BCA method.⁹ After thawing on the day of analysis, 40 - 80 µL of the diluted plasma was analyzed alone for AOC using a PC-controlled BioOrbit Luminometer 1251 (Turku, Finland) with

2,2-azobis (2-amidinopropane) dihydrochloride (AAPH; Polysciences, Warrington, USA) as the peroxy radical generator, and luminol (3-aminophthalhydrazide; Fluka, Milwaukee, USA) as the chemiluminescent indicator reagent. Each sample was analyzed a second time after the addition of 0.6 nM of Trolox as an internal standard, and the net change in the chemiluminescence value was used to calculate the AOC value, expressed as μM of Trolox/mg of plasma protein, or as % of the baseline (pre LPS) plasma value.

Statistics

All results are expressed as the mean \pm standard error of the mean (SEM). The student's *t*-test was used to evaluate differences between the groups. A *P* value of 0.05 or less was considered significant.

RESULTS

When the Sprague-Dawley rats were given 10 mg/kg LPS i.p., they showed neither the expected cardiovascular nor antioxidant perturbations (data not shown).¹⁰ We repeated our efforts at 20, 50, and finally at 100 mg/kg of LPS i.p. before we noticed a behavioral response in unanesthetized rats, which was characterized by decreased spontaneous activity and piloerection. Blood gases and vital signs in the isoflurane-anesthetized rats given 100 mg/kg of LPS i.p. were not significantly different from the control rats over the initial 4-hour period of observation. However, at this

high dosage, a significant increase in arterial plasma AOC was observed for 4 hours after LPS injection (Fig. 1).

Repeating the experiments with lower doses of LPS in this strain and with the replacement of the sampled blood volume with sodium citrate instead of heparin anticoagulant, did not alter the response, which suggested that the lack of cardiovascular changes and the increase in AOC response was not due to the concomitant use of heparin (data not shown).¹¹ Moreover, fasting of animals overnight on chip bedding also did not alter the AOC response at lower LPS doses (data not shown).

Since Wistar rats have been reported to be one

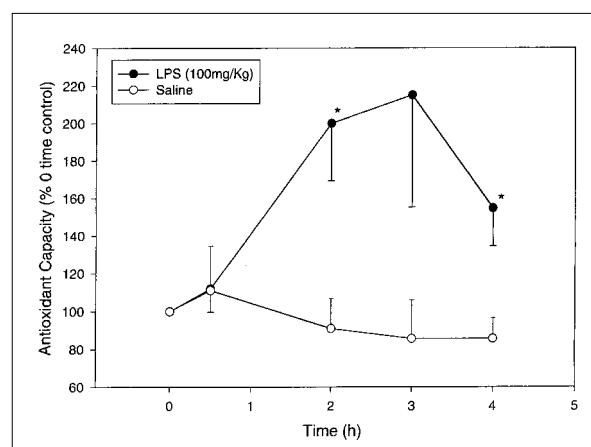


Fig. 1. Sprague-Dawley rats were injected intraperitoneally either 100 mg/kg of LPS or saline (2 mL/kg) at time zero. All values are expressed as % of the zero time value, and brackets indicate the standard errors of the means of 4-6 rats. Plasma AOCs at 2 and 4 hours were significantly different from the comparable values in the control group ($P < 0.05$).

Table 1. Blood Pressure and Plasma Antioxidant Activity after 20 mg/kg Lipopolysaccharide Injection in Sprague-Dawley and Wistar Rats

Rat Strain	Treatment Group	Measured Response to LPS	
		BP	AOC [†]
Sprague-Dawley	Control	81 \pm 2.8	12.7 \pm 0.8
	LPS	112 \pm 22.0	13.4 \pm 2.7
Wistar	Control	101 \pm 6.4	20.0 \pm 3.1
	LPS	100 \pm 8.4	37.0 \pm 2.8*

Each value represents the mean \pm SEM of 4-6 rats.

BP means mean arterial pressure (mmHg).

[†] means plasma antioxidant capacity and expressed as μM of Trolox/mg plasma protein.

* $P < 0.05$ relative to the corresponding species control group.

Table 2. Effects of Non-selective Nitric Oxide Synthase Inhibition on Blood Pressure and Plasma Antioxidant Capacity after 20 mg/kg of Lipopolysaccharide Injection in Sprague-Dawley Rats

Group parameter		Time (min) after LPS		
		0	30	120
L-NAME (n=6)	AOC*	100	98 ± 16	101 ± 16
	BP	115 ± 3 [†]	119 ± 5 [†]	117 ± 5 [†]
Saline (n=6)	AOC*	100	84 ± 20	94 ± 18
	BP	79 ± 7	83 ± 11	81 ± 8

Each value represents the mean ± SEM.

L-NAME, L-N^G-nitroarginine methyl ester hydrochloride.

(50 mg/kg s.c. one and 24 h before the administration of LPS).

*means plasma antioxidant capacity and is expressed as % of the zero time value.

BP represents mean arterial pressure (mmHg).

[†]P < 0.01, statistically significant compared to the control (saline) group.

of the more susceptible rat strains to LPS-induced toxicity,¹ we examined the response of this strain to LPS. At a dose of 20 mg/kg i.p., unanesthetized Wistar rats demonstrated behavior and signs similar to those seen in Sprague-Dawley rats given 100 mg/kg of LPS. In Wistar rats anesthetized with isoflurane and given 20 mg/kg LPS i.p., the maximal increase in AOC was about 80% (Table 1).

Our data suggest that high doses of LPS initially elevate the AOC of arterial blood, at times when no changes in arterial blood gases or arterial pressure were in evidence. Similar changes were noted in both Sprague-Dawley and Wistar rat strains, but at different doses that reflect their differential sensitivities to the LPS induced inflammatory response.

Compared to saline-pretreated controls, the baseline blood pressure of NOS-inhibited rats was significantly elevated, but this pretreatment did not uncover a subsequent hypotensive response, or a increase of AOC in Sprague-Dawley rats given 20 mg/kg of LPS i.p. (Table 2).

DISCUSSION

The current study confirms that Sprague-Dawley rats resist the toxic effects of even very high doses of LPS, and in fact, the rats show increased plasma antioxidant activity, not a decrease as might be expected. This suggests why this rat strain is relatively resistant to the toxicity of LPS in the early period after administration, as evidenced by the absence of changes in blood

pressure, blood gas tension, or base excess measurement. Similar increases in AOC have been reported in Wistar rats over the first 4 hours post-reperfusion of intestinal ischemia models,¹² and this was noted in our experiments with the more sensitive Wistar rats given only 1/5 of the dose of LPS effective at increasing AOC in Sprague-Dawley rats, and which clearly reflected their differential sensitivities to the inflammatory response attributed to LPS.

Although LPS does possess some AOC in our assay, the magnitude of the increase (to 220% of the pre-LPS AOC value) produced by the highest dose of LPS (100 mg/kg i.p.) was probably not due to the absorbed LPS circulating in the blood, since the highest elevation of plasma AOC after LPS (equivalent to 547 μM of Trolox) was much greater than the AOC of the administered LPS (equivalent to 26 nM of Trolox).

L-NAME is a competitive inhibitor of all types of NOS activity, and more potent than another NOS inhibitor, NG-monomethyl-L-arginine.⁷ Dwyer et al observed that brain NOS was inhibited by approximately 50 and 95% by the intraperitoneal administration of 5 and 50 mg/kg, respectively, of L-NAME to Sprague-Dawley rats twice a day for 4 consecutive days.⁷ They also found that a single injection of 50 mg/kg of L-NAME reduced brain NOS activity by 50%, and in their preliminary experiments, after a 90% inhibition of NOS activity with 50 mg/kg of L-NAME, brain NOS activity did not return to normal levels for at least 5 days. In addition, we have previously shown that the subcutaneous administration of 50 mg/kg

of L-NAME 48 h, 24 h, and 30 min before being killed reduces brain NOS activity by more than 90% in our Sprague-Dawley strain rats.¹³ Korb et al also reported that pretreatment with L-NAME (30 mg/kg, i.p., 24 h and 15 min before the administration of LPS) prevented the decrease in plasma fibrinogen level and attenuated most of symptoms of disseminated intravascular coagulation induced by LPS in male Wistar rats.¹⁴ Thus, one would expect significant antagonism of vascular endothelial NOS (eNOS) as well as CNS neuronal NOS (nNOS) to be produced by the peripheral pretreatment of L-NAME 50 mg/kg, 24 h and 1 h before LPS, especially during the first 4 h after LPS administration in our study, although we did not measure NOS activity per se.

It is also known that after LPS injection constitutive eNOS is immediately stimulated, increasing the biosynthesis of nitric oxide (NO), and a progressive reduction¹⁵ and on the other hand, within 1-3 h of LPS administration inducible NOS (iNOS) is expressed in a great variety of cell lines, including hepatocytes and Kupffer cells that produce large amounts of NO, peaking in plasma at 5-6 h.¹⁶⁻¹⁸ In an attempt to determine whether Sprague-Dawley strain resistance to LPS intoxication might be due to their high NOS activity, we treated a group of rats a non-specific NOS inhibitor, L-NAME, which is active against all 3 NOS isoenzymes, but the rats still failed to show any change in blood pressure and plasma AOC at 20 mg/kg LPS. Indeed, even using Wistar rats, a strain that displays gross behavioral signs of intoxication at this dose, no significant change in blood pressure was noted despite the increase in plasma AOC observed. These results may suggest that in our rat strains (including Sprague-Dawley and Wistar rats), the both NOSs (eNOS and iNOS) were not expressed sufficiently to cause hypotension during the initial 4 h period following LPS administration, regardless of the amounts of LPS administered. Moreover, the absence of changes in blood gas tensions and base deficits seems to be another reflection of the resistance of our rat strains to LPS.

Our results suggest that plasma AOC is a poor index of the severity of *E. coli* endotoxin damage in the early period of sepsis in the rat strains. Indeed, the unexpected endotoxin-induced in-

crease in plasma AOC in the early period following administration of LPS may contribute to the rats' resistance to LPS intoxication. Perhaps the deterioration in cardiopulmonary indexes only begins after the AOC has later reduced below its normal level. In the reperfused ischemic intestinal rat model of Slavikova et al,¹² AOC remained elevated 4 hours after the start of reperfusion, which was probably due to the continued release of bacterial debris, whereas, in the present study, AOC was falling at 4 hours after LPS administration, reflecting the continuing clearance of a single dose of LPS or the exhaustion of antioxidant species in plasma.

Even in Slavikova et al.'s study,¹² in which extensive efforts were made to identify the individual antioxidant species responsible for the increased AOC, the best correlation of the increased AOC was with the remaining, unidentified fraction of antioxidant activity. We have no data on the identity of the increased AOC in our study after LPS administration. AOC of plasma has previously been shown to reflect the simultaneous activity of proteins, uric acid, ascorbic acid, and vitamin E.^{19,20} In a study of intestinal ischemia in rats, both ascorbic acid and tocopherol were increased, but their low concentration contributed little to the large absolute increase in AOC.²¹ In a model of myocardial ischemia after endotoxin or lipid A exposure, progressive increases in oxidative stress produced increasing tolerance to subsequent ischemic damage.²² Perhaps AOC defenses called into play by LPS in our studies are similar to those found above in the ischemic intestine and cardiac tissue.^{12,22} This adaptation thus delays developing lipoperoxidation. The results of Pascual et al. suggest that a similar pattern of increase in peroxyl radical trapping capacity has been noted in humans with septic shock, and is largely paralleled by increases in bilirubin and uric acid.²³

In conclusion, our study shows that rat strains are relatively resistant to the development of the toxic signs of LPS in the early period following the administration of LPS, especially in Sprague-Dawley rats. Moreover, endotoxin-induced increase in plasma AOC during the early period after the administration of LPS may contribute to the resistance of the rat to LPS intoxication.

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REFERENCES

1. Fink MP, Heard SO. Laboratory models of sepsis and septic shock. *J Surg Res* 1990;49:186-96.
2. Ben-Shaul V, Sofer Y, Bergman M, Zurousky Y, Grossman S. Lipopolysaccharide-induced oxidative stress in the liver: comparison between rat and rabbit. *Shock* 1999;12:288-93.
3. Kirkeboen KA, Strand OA. The role of nitric oxide in sepsis - an overview. *Acta Anaesthesiol Scand* 1999;43: 275-88.
4. Scott JA, Machoun M, McCormack DG. Inducible nitric oxide synthase and vascular reactivity in rat thoracic aorta: effect of aminoguanidine. *J Appl Physiol* 1996; 80:271-7.
5. Honda K, Kobayashi H, Hataishi R, Hirano S, Fukuyama N, Nakazawa H, et al. Inhaled nitric oxide reduces tyrosine nitration after lipopolysaccharide instillation into lungs of rats. *Am J Respir Crit Care Med* 1999;160:678-88.
6. Park JH, Chang SH, Lee KM, Shin SH. Protective effect of nitric oxide in an endotoxin-induced septic shock. *Am J Surg* 1996;171:340-5.
7. Dwyer MA, Bredt DS, Snyder SH. Nitric oxide synthase: irreversible inhibition by L-N^G-nitroarginine in brain *in vitro* and *in vivo*. *Biochem Biophys Res Commun* 1991;176:1136-41.
8. Visioli F, Galli C. Evaluation of antioxidant capacity by chemiluminescence. *Anal Biochem* 1997;249:244-6.
9. Shihabi Z, Dyer D. Protein analysis with bicinchoninic acid. *Ann Clin Lab Sci* 1988;18:235-9.
10. Hattori Y, Akimoto K, Nakanishi N, Kasai K. Glucocorticoid regulation of nitric oxide and tetrahydrobiopterin in a rat model of endotoxic shock. *Biochem Biophys Res Commun* 1997;240:298-303.
11. Wang P, Ba ZF, Chaudry IH. Chemically modified heparin improves hepatocellular function, cardiac output, and microcirculation after trauma-hemorrhage and resuscitation. *Surgery* 1994;116:169-76.
12. Slavikova H, Lojek A, Hamar J, Duskova M, Kubala L, Vondracek J, et al. Total antioxidant capacity of serum increased in early but not late period after intestinal ischemia in rats. *Free Radic Biol Med* 1998;25:9-18.
13. Mueller RA, Hunt R. Antagonism of ketamine-induced anesthesia by an inhibitor of nitric oxide synthesis: A pharmacokinetic explanation. *Pharmacol. Biochem. Behav.* 1998;60:15-22.
14. Korbut R, Warner TD, Gryglewski RJ, Vane JR. The effect of nitric oxide synthase inhibition on the plasma fibrinolytic system in septic shock in rats. *Br J Pharmacol* 1994;112:289-91.
15. Wang P, Zheng FB, Chaudry IC. Nitric oxide. To block or enhance its production during sepsis? *Arch Surg* 1994;129:1137-43.
16. Hortelano S, Genaro AM, Bosca L. Phorbol esters induce nitric oxide synthase activity in rat hepatocytes. Antagonism with the induction elicited by lipopolysaccharide. *J Biol Chem* 1992;267:24937-40.
17. Szabo C, Mitchell JA, Thiemermann C, Vane JR. Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock. *Br J Pharmacol* 1993;108:786-92.
18. Wallis G, Brackett D, Lerner M, Kotake Y, Bolli R, McCay PB. *In vivo* spin trapping of nitric oxide generated in the small intestine, liver, and kidney during the development of endotoxemia: a time-course study. *Shock* 1996;4:274-8.
19. Wayner DD, Burton GW, Ingold KU, Locke S. Quantitative measurement of the total, peroxy radical trapping antioxidant capability of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins. *FEBS Lett* 1985;187:33-7.
20. Uotila JT, Kirkkola A-L, Rovarius M, Turnala RJ, Metsaketela T. The total peroxy radical-trapping ability of plasma and cerebrospinal fluid in normal and pre-clamptic patients. *Free Radic Biol Med* 1994;16:581-90.
21. Osborne DL, Aw TY, Cepnaskas G, Kvietys PR. Development of ischemia/reperfusion tolerance in the rat small intestine. An epithelium-independent event. *J Clin Invest* 1994;94:1910-8.
22. Maulik N, Watanabe M, Engelman DT, Engelman RM, Das DK. Oxidative stress adaptation improves postischemic ventricular recovery. *Mol Cell Biochem* 1995;144:67-74.
23. Pascual C, Karzai W, Meier-Hellman A, Oberhoffer M, Horn A, Bredle D, et al. Total plasma antioxidant capacity is not always decreased in sepsis. *Crit Care Med* 1998;26:705-9.