JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 117–125
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Received July 22, 2004
Accepted September 27, 2004
DOI 10.1211/0022357055137
ISSN 0022-3573

Antioxidant eugenosedin-A protects against lipopolysaccharide-induced hypotension, hyperglycaemia and cytokine immunoreactivity in rats and mice

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Abstract

Eugenosedin-A has been demonstrated to possess α/β-adrenoceptor and serotonergic receptor blocking activities. We have investigated by what mechanisms eugenosedin-A prevents lipopolysaccharide (LPS)-induced hypotension, vascular hyporeactivity, hyperglycaemia, oxidative injury or inflammatory cytokines formation in rats. Intravenous administration of eugenosedin-A, trazodone, yohimbine (1 mg kg^{-1}), aminoguanidine or ascorbic acid (15 mg kg^{-1}) normalized LPS (10 mg kg^{-1})-induced hypotension. Pretreatment with eugenosedin-A or the other agents 30 min before LPS injection reduced aortic hyporeactivity. LPS-induced increases in plasma interleukin-1β (IL-β), IL-6, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and blood glucose levels were significantly inhibited by eugenosedin-A (1 mg kg^{-1}, i.v.). The same dose of trazodone, a chloropiperazinylbenzene-type antidepressant, and yohimbine, an α_2-adrenoceptor antagonist, reduced IL-1β and TNF-α, but it could not inhibit hyperglycaemia. Aminoguanidine, an inducible nitric oxide synthase (iNOS) inhibitor, and ascorbic acid, an antioxidant, decreased IL-1β, TNF-α contents and hyperglycaemia. Eugenosedin-A and the other agents inhibited Fe^{2+}-ascorbic acid-induced peroxidation in rat cortex, indicating that those agents had antioxidant effects, with the exception of aminoguanidine. In free radical scavenged experiments, eugenosedin-A and ascorbic acid eliminated peroxyl radicals. All test agents inhibited the LPS-induced increase of malondialdehyde (MDA) content in rat brain homogenates. When mice were administered an intraperitoneal injection of LPS alone, mortality occurred from 4 to 16 h, after which time all were dead. However, eugenosedin-A significantly prolonged the survival time after LPS injection, suggesting that eugenosedin-A protected against LPS-induced cardiovascular dysfunction, hyperglycaemia, tissue injury and inflammatory cytokine production. This was attributable mainly to the antioxidant and peroxyl radical scavenged effects of eugenosedin-A, and which may be, at least in part, due to its blockade on α/β-adrenergic and serotonergic receptors.

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Funding: This study was partly supported by grants NSC-89-2320-B-037-056-M59 (I.-J. Chen), NSC-91-2745-B-037-002 (H.-W. Liu) and NSC-92-2320-B-037-025 (B.-N. Wu) from the National Science Council, Taiwan, and Syn-Tech Chem. & Pharm. Co., Ltd (Tainan, Taiwan).

Introduction

Severe sepsis resulting from Gram-negative bacterial infections is a major clinical problem. Several pharmacological agents have been used to protect against bacterial lipopolysaccharide (LPS)-induced hypotension and inflammation, including the pressor agent adrenergic agonists to prevent hypotension, and corticosteroids to reduce inflammation and associated tissue damage. By contrast, α_2-adrenoceptor blockers provided some protection in rats against bacterial LPS-induced hyperglycaemia, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), corticosteroid release and mortality (Haskó et al 1995; Fessler et al 1996). Moreover, serotonin (5-HT) has been involved in LPS-induced cytokine formation (Bertini et al 1993; Maes et al 1999). However, to date, similar protective functions provided by trazodone-like antidepressants, with α_2-adrenoceptors and 5-HT blocking activities, have not been investigated thoroughly.

LPS-induced inflammatory cytokines, including TNF-α, interleukin-1 (IL-1) and interferon (IFN), can be regulated by blocking α_2-adrenoceptors, which are involved in

the balance between noradrenergic and serotonergic systems in central neurons (Shen et al 1999). Despite the importance of LPS-induced inflammatory response, many aspects of LPS-induced dysfunction remain poorly understood. To date, the relationship between LPS-induced hypotension and high mortality has not been resolved. LPS is known to affect cerebral neurotransmission and induce CNS toxicity (Molina-Holgado & Guaza 1996). In addition, α_2- and β-adrenoceptors on macrophages can be activated by the endogenous noradrenaline (norepinephrine), which is released from noradrenergic varicosities and by adrenergic drugs. It has been suggested that these increases can regulate LPS-induced production of cytokines (Szelényi et al 2000). That fact that α_2-adrenoceptor blocking agents attenuated LPS-induced blood pressure depression in rats has been cited as evidence that inflammatory cytokines play an important role in hypotension (Koyama 1984). 5-HT is a neurotransmitter and belongs to a vasoactive amine released from platelets and lymphocytes/monocytes following stimulation by platelet activating factors and lectins/IFN-γ, respectively (Finocchiaro et al 1988). Pharmacological and molecular analyses have confirmed the presence of 5-HT_{1A} and 5-$HT_{2A/C}$ receptors on activated human immunocytes and specific 5-HT transporter sites on macrophages/lymphocytes (Aune et al 1994).

2-Chloro-1-piperazinylbenzene (CPB) has been demonstrated to have α_2-adrenoceptors and 5-HT antagonistic activities (Malomvolgyi et al 1991), and this was further confirmed with eugenosedin-A (Shen et al 2004). In addition, aryloxypropanolamines, especially those that are isoeugenol-based, have anti-oxidizing activities in addition to their β-adrenoceptor blocking effects (Huang et al 2001). With the view of developing an α/β-adrenoceptor and 5-HT receptor antagonist with enhanced anti-oxidizing, oxidative injury reducing and inflammatory cytokine inhibiting activities, eugenosedin-A (4-{2-hydroxy-3-[1-(2-chlorophenyl-piperazinyl)]-propoxy}-3-methoxy-1-propylenyl-benzene) was synthesized by combining isoeugenol-based aryloxypropanolamine and CPB (Shen et al 2004). An antidepressant trazodone, also with the CPB moiety, was used as a reference to evaluate associated activities and LPS-induced hypotension, hyperglycaemia and cytokine formation.

We have examined the antioxidant activity, peroxyl radical scavenging, and inhibition of oxidative injury of eugenosedin-A in rats. To examine the protective activity of eugenosedin-A with respect to the effects of LPS, we performed a survival test on mice.

Materials and Methods

Animals

Wistar rats and ICR mice were provided by the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). They were housed under conditions of constant temperature and controlled illumination (lights on between 0730 and 1930 h). Food and water were freely available. The study was approved by the Animal Care and Use Committee of Kaohsiung Medical University.

Drugs and chemicals

LPS (*Escherichia coli* serotype 0127:B8) and yohimbine were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Trazodone was kindly supplied from Lotus Medical Supply Co. (Taipei, Taiwan). Eugenosedin-A was synthesized in this laboratory. All drugs and reagents were dissolved in distilled water unless otherwise noted. LPS was dissolved in sterile 0.9% saline. Eugenosedin-A, trazodone and yohimbine were dissolved in 10% absolute alcohol and 10% propylene glycol, and further dilutions were made in distilled water.

LPS-induced hypotension

The experiment was carried out as described by Wu et al (1994). In brief, Wistar rats (250–300 g) were anaesthetized with urethane (1.5 g kg^{-1}, i.p.). Following tracheal cannulation, systemic arterial blood pressure was recorded from the femoral artery by a pressure transducer (model P10EZ; Spectramed, Oxnard, CA) connected to a recorder (GOULD, Valley View, Ohio, Model P50). Body temperature was maintained at 37°C by an electric heating pad. A femoral vein was cannulated for intravenous injection of test agents and LPS (10 mg kg^{-1}). The intravenous volume of test agents into each rat was 0.5 mL kg^{-1}. Eugenosedin-A, yohimbine, trazodone (1 mg kg^{-1}, i.v.), aminoguanidine or ascorbic acid (15 mg kg^{-1}, i.v.) was administered 30 min before LPS injection, after which changes in blood pressure were recorded for 5 h.

Hyporeactivity of rat thoracic aorta after LPS injection

Aortic rings and atria were mounted at 1 g resting tension on stainless steel hooks in a 10-mL organ bath, bathed at 37°C in physiological solution (mM: NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11) and aerated with a 95% O_2 and 5% CO_2 mixture. Isometric tension of aortic rings was monitored by a force displacement transducer (UGO BASILE, Model 7004, Italy) (Wu et al 2001). For measuring LPS-induced vascular hyporeactivity, rats were pretreated with eugenosedin-A or other agents for 30 min before LPS (10 mg kg^{-1}) injection. At 1, 3 and 5 h after the injection of vehicle or LPS, thoracic aortas were obtained from sham-treated controls as well as from endotoxaemic rats. The vessels were cleared of adhering fat and connective tissue, and cut into 3–4-mm lengths. The endothelium was removed by gently rubbing the intimal surface of the vessel (Furchgott & Zawadzki 1980). Later, the lack of a relaxation to acetylcholine (10^{-6} M) was considered as evidence that the endothelium had been removed. Phenylephrine ($10^{-8} \sim 10^{-4}$ M) was added to the bath cumulatively. The pD_2 values were calculated as a percentage of the increases in force induced by phenylephrine and estimated vascular contraction activity.

Plasma cytokine immunoreactivity and blood glucose

Blood was collected from a venous cannula, injected into ice-cold heparinized Eppendorf tubes and centrifuged at 400 *g* for 10 min at 4°C. Plasma supernatant was stored at −70°C until analysis. Solid phase enzyme immunoassay that specifically detects murine IL-1β, IL-6, IFN-γ and TNF-α was used; the detection limit was $> 10\,pg\,mL^{-1}$ (Endogen, US). Pretreatment with eugenosedin-A or other agents was performed 30 min before intravenous injection of LPS ($10\,mg\,kg^{-1}$). Blood glucose was measured with a glucose test strip (Glucotide, Bayer, US) at 1, 3 and 5 h.

Inhibition on LPS-induced oxidative injury

Rat brains were dissected after LPS-injection experiments and homogenates were made in 0.9% saline containing $10\,mg$ tissue mL^{-1}. The samples were centrifuged at 600 *g*, using Dupont Sorvall Combi Plus ultracentrifuge, and the supernatant was used for MDA (malondialdehyde) estimation (MDA-586, Oxis Research, US) (Kheir-Eldin et al 2001).

Inhibition of lipid peroxidation and peroxyl radical scavenging activity

Rat brain homogenate was made in 0.9% saline containing $10\,mg$ tissue mL^{-1}. The rates of membrane lipid peroxidation were measured by the formation of thiobarbituric acid (TBA)-reactive substance (TBARS). Rat brain homogenates (1 mL) were incubated at 37°C for 5 min with 10 μL of test compound or vehicle. Lipid peroxidation was initiated by the addition of 0.1 mL 0.25 mM $FeCl_2$ and 1 mM ascorbic acid (Huang et al 1999). After 30-min incubation, the reaction was stopped by the addition of 0.1 mL 0.2% butylated hydroxytoluene (BHT). TBA reagent was then added, and the mixture was heated for 30 min in a boiling water bath. TBARS was extracted by n-butanol and measured at 532 nm. The amount of TBARS was quantified using the linear regression obtained from MDA standards.

The scavenging ability of the test compounds on aqueous peroxyl radicals was determined by the method described by Tsuchiya et al (1992). The reaction mixture (2 mL) consisted of 0.5 nM β-phycoerythrin, 25 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) in 75 mM sodium phosphate buffer (pH 7.4). The stoichiometric factors of the test compounds with hydrophilic peroxyl radicals were calculated according to the lag time during which fluorescence loss was protected (excitation at 540 nm and emission at 570 nm). Ascorbic acid was used as a positive control.

Survival study

The experimental protocol was slightly modified from Kang et al (2002). Mice were randomly assigned to six groups (n = 10 each). LPS ($10\,mg\,kg^{-1}$, i.p.) was injected in the presence of vehicle or test agents and survival was monitored every 4 h for two days. Different groups of animals received vehicle and test agents including eugenosedin-A, yohimbine, trazodone ($1\,mg\,kg^{-1}$, i.p.), aminoguanidine or ascorbic acid ($15\,mg\,kg^{-1}$, i.p.) 30 min before LPS ($10\,mg\,kg^{-1}$, i.p.) injection. Increases in survival rate at each observation time indicated the benefits of treatment.

Statistical evaluation

Results are expressed as mean ± s.e. Statistical differences were determined by independent and paired Student's *t*-test in unpaired and paired samples. Whenever a control group was compared with more than one treated group, the one-way analysis of variance or two-way repeated measures analysis of variance was used. When the analysis of variance manifested a statistical difference, Dunnett's or Student–Newman–Keuls test was applied. $P < 0.05$ was considered to be significant. Analysis of data and plotting of figures were done with the aid of software (SigmaStat: Version 2.03 and SigmaPlot: Version 8.0, Chicago, IL) run on an IBM-compatible computer.

Results

Normalization of LPS-induced hypotension

Acute intravenous injection of eugenosedin-A, yohimbine or trazodone ($1\,mg\,kg^{-1}$) produced hypotensive effects in anaesthetized Wistar rats, but these effects were completely recovered at 60 min. By contrast, ascorbic acid or an inducible nitric oxide synthase (iNOS) inhibitor, aminoguanidine, did not affect the blood pressure (Figure 1A). On the other hand, a single injection of LPS ($10\,mg\,kg^{-1}$, i.v.) produced biphasic hypotensive effects: an immediate and transient decrease in blood pressure that was followed by partial recovery within 30 min and was then followed by prolonged hypotension. The first immediate depressor response was affected by pretreatment with eugenosedin-A, yohimbine, trazodone ($1\,mg\,kg^{-1}$, i.v.), aminoguanidine or ascorbic acid ($15\,mg\,kg^{-1}$, i.v.) 30 min before LPS injection. Although LPS-induced depressor responses had not fully recovered after 30 min, all of them prevented further development of the second prolonged depressor response and normalized blood pressure changes within 5 h (Figure 1B). After 5 h, the survival rate of the LPS-treated group was only 30%, but that of the eugenosedin-A-pretreated group was above 70% (unpublished data).

Protective effects against LPS-induced vascular hyporeactivity

Isolated aortas from LPS-treated rats were hyporeactive to phenylephrine ($10^{-8} \sim 10^{-4}$ M). However, 1 h after administration of LPS, the vessel showed effects similar to that of LPS-untreated groups (unpublished data). By pretreatment with eugenosedin-A ($1\,mg\,kg^{-1}$, i.v.) 30 min before LPS injection, the aortic hyporeactivity could be prevented. Compared with yohimbine, trazodone, aminoguanidine

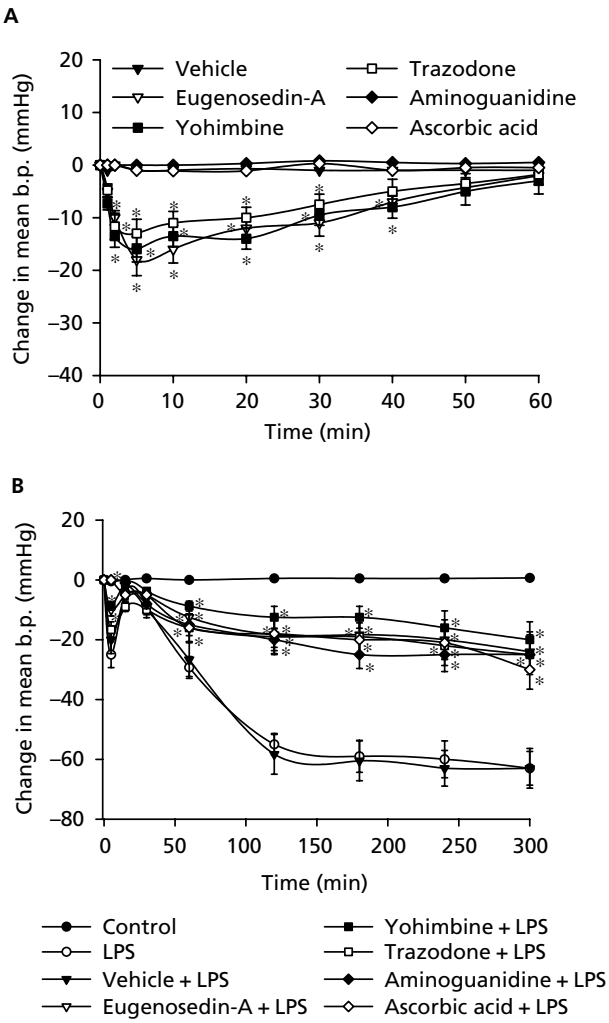

Figure 1 Effects of eugenosedin-A, yohimbine, trazodone (1 mg kg^{-1}, i.v.), aminoguanidine or ascorbic acid (15 mg kg^{-1}, i.v.) in the absence (A) and presence (B) of LPS (10 mg kg^{-1}, i.v.) on mean arterial blood pressure (mean b.p.) in Wistar rats, anaesthetized with urethane. Saline was used as control. Vertical bars represent s.e. change from the basal line value, which was 115 ± 10 mmHg for mean b.p. Results are expressed as mean $\pm$ s.e. Each point represents the mean of six to eight rats. A, $^*P < 0.05$ compared with vehicle; B, $^*P < 0.05$ compared with the vehicle plus LPS, (repeated-measures analysis of variance followed by Student–Newman–Keuls test).

and ascorbic acid, eugenosedin-A was more effective in protecting from LPS-induced hyporeactivity of the aorta while at 3 or 5 h. The abilities of eugenosedin-A, ascorbic acid and aminoguanidine against LPS-induced vessel hyporeactivity at 5 h were better than those of yohimbine or trazodone (Table 1).

Inhibition of LPS-induced cytokine immunoreactivity and hyperglycaemia

The basal values of IL-1β, IL-6, IFN-γ and TNF-α were 0.062 ± 0.011, 0.931 ± 0.045, 0.002 ± 0.001 and 0.090 ± 0.002 ng mL^{-1}, respectively. The immunoreactivity of IL-1β, IL-6, IFN-γ and TNF-α was increased 1, 3 and 5 h after LPS administration (10 mg kg^{-1}, i.v.) (Table 2). None of the administered agents significantly reduced LPS-induced increases in these cytokines at 1 and 5 h. However, pretreatment with eugenosedin-A (1 mg kg^{-1}, i.v.) significantly attenuated those of LPS-induced releases of cytokines at 3 h. Yohimbine, trazodone or aminoguanidine also significantly lessened the increases of IL-1β and TNF-α at 3 h, whereas ascorbic acid only markedly affected the IL-1β (Table 2).

The basal blood glucose level in rats was 126 ± 5 mg dL^{-1}. When LPS was administered, hyperglycaemia occurred at 1 and 3 h, followed by progressive hypoglycaemia at 5 h. Eugenosedin-A, aminoguanidine and ascorbic acid but not yohimbine or trazodone reduced the initial hyperglycaemic response to LPS at 1 and 3 h. By contrast, none of them could reverse the hypoglycaemic response at 5 h (Table 3).

Table 1 The pD_2 values were obtained from phenylephrine-induced vasocontractions in the absence and presence of LPS in rat aorta

Treatment	pD_2 (3 h)	pD_2 (5 h)
None	7.35 ± 0.19	7.36 ± 0.20
Vehicle	7.32 ± 0.22	7.31 ± 0.19
Vehicle + LPS	5.72 ± 0.27	4.94 ± 0.13
Eugenosedin-A + LPS	7.16 ± 0.18*	6.29 ± 0.11*
Yohimbine + LPS	6.73 ± 0.33*	3.44 ± 0.22
Trazodone + LPS	6.29 ± 0.21*	3.99 ± 0.16
Aminoguanidine + LPS	6.81 ± 0.17*	5.44 ± 0.31*
Ascorbic acid + LPS	6.71 ± 0.13*	5.11 ± 0.22

The statistical analysis was performed using repeated-measures analysis of variance followed by Student–Newman–Keuls test. Each test agent-treated group was compared with the vehicle plus LPS group at 3 or 5 h. $^*P < 0.05$ compared with the vehicle plus LPS ($n = 6$–8).

Inhibition of LPS-induced oxidative injury

LPS injection caused a significant increase in brain MDA at 3 and 5 h, compared with the basal concentration (0.56 ± 0.06 μM), but not at 1 h. Eugenosedin-A, yohimbine, trazodone, aminoguanidine and ascorbic acid all significantly attenuated LPS-induced elevations of MDA formation at 3 and 5 h (Figure 2).

Antioxidant and peroxyl radical scavenging activities

To eliminate the possibility that eugenosedin-A and other test compounds were interfering with the assay, the agents were added directly to MDA standard before the TBA reagent was added. The abilities of eugenosedin-A and other test compounds to inhibit lipid peroxidation in rat brain homogenate are compared in Table 4. Eugenosedin-A dose-dependently inhibited Fe^{2+}-ascorbic acid-induced lipid peroxidation in rat brain homogenate with an IC50

Table 2 Intravenous injection of eugenosedin-A, yohimbine, trazodone (1 mg kg^{-1}), aminoguanidine or ascorbic acid (15 mg kg^{-1}) on LPS-induced cytokine formation at 1, 3 and 5 h

Treatment	1 h				3 h				5 h			
	IL-1β	IL-6	IFN-γ	TNF-α	IL-1β	IL-6	IFN-γ	TNF-α	IL-1β	IL-6	IFN-γ	TNF-α
Vehicle + LPS	0.158 (0.012)	0.844 (0.020)	0.013 (0.002)	0.227 (0.027)	0.751 (0.023)	3.443 (0.300)	0.231 (0.015)	0.204 (0.026)	0.740 (0.019)	3.544 (0.330)	1.478 (0.330)	0.110 (0.019)
Eugenosedin-A + LPS	0.156 (0.018)	0.992 (0.018)	0.014 (0.003)	0.248 (0.036)	0.275* (0.010)	2.400* (0.150)	0.130* (0.026)	0.044* (0.017)	0.665 (0.020)	3.325 (0.210)	1.430 (0.210)	0.100 (0.026)
Yohimbine + LPS	0.146 (0.040)	1.000 (0.040)	0.015 (0.003)	0.271 (0.023)	0.532* (0.012)	3.200 (0.269)	0.210 (0.012)	0.081* (0.024)	0.819 (0.019)	3.267 (0.260)	1.670 (0.260)	0.149 (0.021)
Trazodone + LPS	0.166 (0.015)	0.900 (0.015)	0.013 (0.002)	0.278 (0.027)	0.388* (0.020)	3.172 (0.289)	0.261 (0.030)	0.115* (0.016)	0.723 (0.015)	3.283 (0.290)	1.623 (0.290)	0.144 (0.024)
Aminoguanidine + LPS	0.174 (0.015)	0.942 (0.015)	0.019 (0.012)	0.217 (0.036)	0.557* (0.011)	3.169 (0.260)	0.245 (0.011)	0.110* (0.015)	0.646 (0.018)	3.325 (0.341)	1.685 (0.341)	0.143 (0.016)
Ascorbic acid + LPS	0.145 (0.030)	0.935 (0.030)	0.014 (0.003)	0.255 (0.026)	0.284* (0.025)	2.800 (0.381)	0.213 (0.025)	0.178 (0.025)	0.771 (0.022)	3.285 (0.350)	1.635 (0.350)	0.146 (0.025)

Values of cytokine formation are in ng mL^{-1}; each value in parenthesis indicates the s.e. (n = 8). *$P < 0.05$ compared with the vehicle plus LPS (two-way analysis of variance followed by Dunnett's test).

Table 3 Intravenous injection of eugenosedin-A, yohimbine, trazodone (1 mg kg^{-1}), aminoguanidine or ascorbic acid (15 mg kg^{-1}) on LPS-induced changes of the blood glucose level at 1, 3 and 5 h

Treatment	Blood glucose (mg dL^{-1})		
	1 h	3 h	5 h
Vehicle + LPS	168 ± 8.1	183 ± 11.6	44 ± 2.9
Eugenosedin-A + LPS	128 ± 5.9*	139 ± 5.8*	59 ± 7.5
Yohimbine + LPS	155 ± 4.5	185 ± 3.9	50 ± 8.3
Trazodone + LPS	148 ± 6.4	190 ± 9.2	66 ± 6.1
Aminoguanidine + LPS	126 ± 3.3*	130 ± 4.8*	53 ± 4.8
Ascorbic acid + LPS	127 ± 4.1*	128 ± 4.7*	41 ± 3.9

Each value represents the mean of eight rats. *$P < 0.05$ compared with the vehicle plus LPS, (two-way analysis of variance followed by Dunnett's test).

of 2.68 ± 0.27 μM (n = 6). The potency of eugenosedin-A was approximately 4-, 23- and 3-times that of yohimbine, trazodone and ascorbic acid, respectively (Table 4).

Exposure of β-phycoerythrin to AAPH-derived aqueous peroxyl radicals induced a transient decay of fluorescent intensity. Eugenosedin-A (0.1, 1, 10 μM) and ascorbic acid (10 μM) produced a concentration-dependent decrement of fluorescence and prolongation of the lag time (Figure 3). We observed that yohimbine and trazodone did not significantly scavenge peroxyl radicals (unpublished data).

Survival rate of mice treated with LPS

Figure 4 shows that after intraperitoneal injection of LPS (10 mg kg^{-1}) none of the mice treated with vehicle survived beyond 16 h. In contrast, pretreatment with eugenosedin-A, yohimbine, trazodone (1 mg kg^{-1}, i.p.) or aminoguanidine (15 mg kg^{-1}, i.p.) 30 min before LPS administration significantly prevented mortality and prolonged survival. However, ascorbic acid (15 mg kg^{-1}, i.p.) did not improve the survival rate in LPS-treated mice.

Discussion

Eugenosedin-A, which has a similar CPB structure to that of trazodone, produces comparable serotonergic and adrenergic antagonistic effects. We demonstrated previously (Shen et al 2004) that eugenosedin-A possessed α_1/α_2- and β_1- but not β_2-adrenoceptor antagonistic activities, and serotonergic receptor blocking actions. We know that β-adrenoceptors may be down-regulated and unable to respond fully to catecholamine-derived β-adrenoceptor agonist and drug-derived β-adrenergic antagonist activity during sepsis. In this study, we have clarified that the protective effects of eugenosedin-A against LPS-induced cardiovascular dysfunction, hyperglycaemia, oxidative injury and inflammatory cytokine formation could be attributed to its serotonergic and α/β-adrenoceptor blocking properties and specific antioxidant activities.

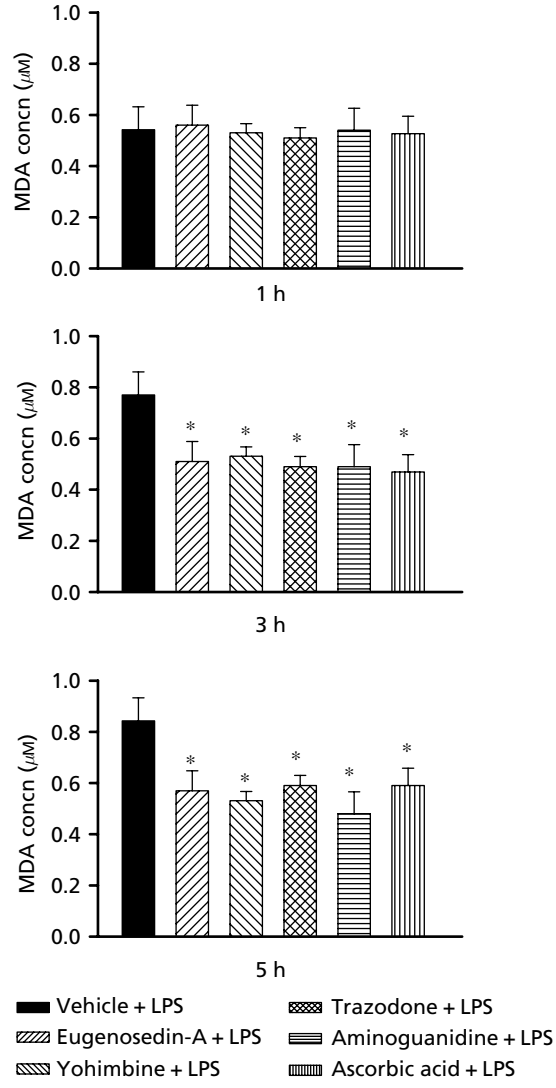

Figure 2 Pretreatment with eugenosedin-A, yohimbine, trazodone (1 mg kg^{-1}, i.v.), aminoguanidine or ascorbic acid (15 mg kg^{-1}, i.v.) on the LPS-induced increases of MDA formation at 1, 3 and 5 h after administration. Each value represents the mean of eight rats. *$P < 0.05$ compared with the vehicle plus LPS (two-way analysis of variance followed by Dunnett's test).

Table 4 Concentrations for fifty percent inhibition (IC50) required in inhibiting lipid peroxidation initiated by Fe^{2+}-ascorbic acid in rat brain homogenates

Compound	IC50 (μM)	Potency
Eugenosedin-A	2.68 ± 0.27	1
Yohimbine	11.09 ± 0.35	0.24
Trazodone	60.61 ± 0.76	0.04
Ascorbic acid	7.15 ± 0.14	0.37

IC50 values were calculated from the mean of six independent experiments.

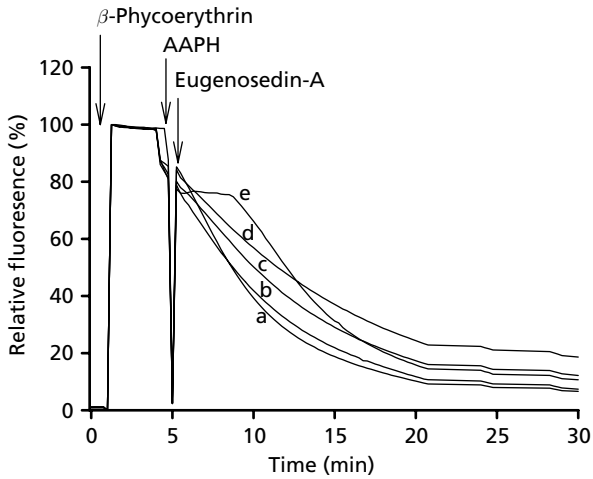

Figure 3 Effects of eugenosedin-A and ascorbic acid on hydrophilic peroxyl radical-induced degradation of β-phycoerythrin and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). Fluorescence intensity of β-phycoerythrin was measured by excitation at 540 nm and emission at 570 nm. The arrow indicates the moment when vehicle (a, 5% alcohol), eugenosedin-A (b, 0. 1; c, 1; d, 10 μM), or ascorbic acid (e, 10 μM) was added to the reaction mixture. One of the representative tracings (n = 6) is shown.

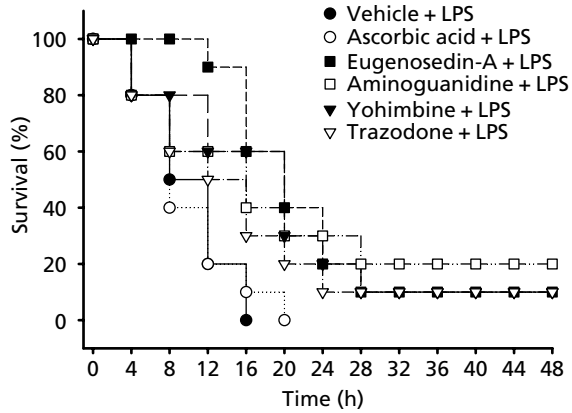

Figure 4 Survival rates for mice challenged with 10 mg kg^{-1} LPS intraperitoneally and treated with vehicle, eugenosedin-A, yohimbine, trazodone (1 mg kg^{-1}, i.p.), aminoguanidine or ascorbic acid (15 mg kg^{-1}, i.p.) 30 min before LPS, n = 10 for each group.

Previous studies have demonstrated a neuro-immune link that enables stress-associated noradrenaline to regulate macrophage-derived TNF via α-adrenergic receptor interactions. Noradrenaline and α_2-adrenergic agonists have been shown to augment LPS-induced TNF production. This augmentation was prevented by the α_2-adrenergic antagonist yohimbine (Spengler et al 1990; Fessler et al 1996). Nickola et al (2001) pointed out that a reciprocally permissive interaction occurred between TNF-α and α-adrenoceptor activation. Additionally, there is some evidence that antidepressants may suppress the release of cytokines, such as IL-1β and IL-6 by activated monocytes and IL-2 and IFN-γ by activated T cells (Maes et al 1999). 5-HT, 5-HT$_{1A}$, 5-HT$_{2A/2C}$ receptor antagonists, selective serotonin reuptake inhibitors (SSRIs) and depletion of intracellular 5-HT were able to suppress various aspects of the inflammatory response system (Smejkal-Jagar & Boranic 1994). In this study, intravenous LPS produced a biphasic reduction in blood pressure in anaesthetized rats. The second prolonged hypotensive response induced by LPS, which was due to an enhanced formation of NO by iNOS (Szabó et al 1993), was attenuated by eugenosedin-A, yohimbine, trazodone, aminoguanidine and ascorbic acid. Although when administered alone eugenosedin-A, yohimbine and trazodone all exhibited hypotensive effects in control rats, the blood pressure changes completely recovered 1 h after drug administration. By contrast, the severe delayed and prolonged hypotension elicited by LPS was sustained for more than 5 h. From the results, notably, the hypotension produced by eugenosedin-A, yohimbine or trazodone alone was strikingly different from the LPS-induced circulatory failure associated hypotension. Additionally, they all significantly attenuated LPS-induced productions of IL-1β and TNF-β at 3 h. Not only IL-1β and TNF-α but also IL-6 and IFN-γ were lowered by eugenosedin-A at 3 h (Table 2). We suggest that the restoration of LPS-induced hypotensive responses by eugenosedin-A appeared to block α_2-adrenoceptors and/or 5-HT receptors, to reduce cytokine production, and, at least in part, to inhibit LPS-induced NO production that was mediated by iNOS activation.

Hyperglycaemia in the early phase of sepsis is caused by a decrease in peripheral tissue glucose uptake relative to the rate of glucose production. In contrast, hypoglycaemia in the severe septic condition occurs because the rate of glucose use exceeds the rate of production (Lang 1992). In this study, LPS-induced hyperglycaemia at 1 or 3 h was inhibited by eugenosedin-A. However, eugenosedin-A could not prevent the hypoglycaemia induced by LPS at 5 h. On the other hand, atenolol, a selective β-adrenergic blocker, does not alter the glucose metabolic response suffered from infections. Under septic conditions, a non-selective β-adrenoceptor blocker propranolol prevented the increases of glucose production (Lang 1992). Since eugenosedin-A is more selective for β-adrenoceptors, but not β_2-adrenoceptors (Shen et al 2004), we suggest that its protection against LPS-induced hyperglycaemia could be involved in the blockade of α-adrenoceptors and/or inhibition of gluconeogenesis, leading to the reduction of glucose levels in the early phase of sepsis.

The overproduction of NO was suggested to be responsible for the vascular relaxation and hypotension seen in states of sepsis and endotoxaemia. The expression of iNOS in animals with endotoxaemia was associated with the level of cytokines such as TNF-α, IL-1 and IFN-γ (Moncada et al 1991). Sepsis and endotoxaemia induced the cellular production of an excessive amount of reactive oxygen species, superoxides in particular, which have been implicated in the potentiation of iNOS production in cells. Many reports have shown that iNOS inhibitors and antioxidants can ameliorate LPS-induced vascular

hyporesponsiveness (Wu et al 1995; Briones et al 2000; Wu et al 2002). In this study, we ascertained that eugenosedin-A, yohimbine, trazodone, aminoguanidine and ascorbic acid could restore the vascular hyporeactivity in LPS-treated rats. Among these, we observed that eugenosedin-A was more effective in protecting from LPS-induced hyporeactivity of the aorta. Our in-vivo and in-vitro results suggested that eugenosedin-A could provide beneficial effects to prevent vascular hyporeactivity and to improve the survival and haemodynamic alterations caused by endotoxic shock. In addition, our results showed that LPS administration enhanced the production of brain MDA, which is in agreement with the in-vivo study of Kheir-Eldin et al (2001). Several mechanisms were postulated to explain this phenomenon, including the enhanced formation of cytokines and associated release of reactive oxygen species and nitric oxide from microglial cells (Richter & Kass 1991; Moghaddam 1993; Woodroofe 1995). Thus, we suggest that the antioxidant actions of eugenosedin-A could provide a protective mechanism against inflammatory cytokines. The generation of free radicals in biological systems contributes to oxidative stress, including inflammation. Eugenosedin-A possesses free radical scavenging and lipid peroxidation inhibitory properties, while yohimbine and trazodone have only modest anti-peroxidation. Additionally, eugenosedin-A reduced the inflammatory cytokine formation by LPS that was more potent than that of yohimbine or trazodone. In this regard, the relationship between the antioxidant and antihypotensive activity of eugenosedin-A could contribute to its inhibition of free radical-induced lipid peroxidation, prevention of oxidative injury to vascular endothelial cells, and its reduction of inflammatory cytokines.

Conclusion

Eugenosedin-A antagonized the α/β-adrenoceptor and the 5-HT receptor, in particular, with antioxidant activity, and thus it prevented the early stage of LPS-induced hypotensive response, vascular hyporeactivity, hyperglycaemia and tissue-injury-related cytokine formation. Although eugenosedin-A could not successfully reduce LPS-induced death in mice, it did significantly prolong the survival time. We suggest that eugenosedin-A could provide benefits in improving the survival rate and preventing the circulatory failure associated with endotoxic shock in this animal model.

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