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Eugenosedin-A amelioration of lipopolysaccharide-induced up-regulation of p38 MAPK, inducible nitric oxide synthase and cyclooxygenase-2

Kuo-Ping Shen, Shu-Fen Liou, Su-Ling Hsieh, Ing-Jun Chen and Bin-Nan Wu

Abstract

In this study, we investigate the protective effects of eugenosedin-A on p38 mitogen-activated protein kinase (MAPK), inflammatory nitric oxide (NO) and cyclooxygenase-2 (COX-2) pathways in a rat model of endotoxin shock. Rats were pretreated with eugenosedin-A, trazodone, yohimbine (1 mg kg^{-1}, i.v.), aminoguanidine or ascorbic acid (15 mg kg^{-1}, i.v.) 30 min before endotoxin challenge. Endotoxaemia was induced by a single i.v. injection of lipopolysaccharide (LPS, 10 mg kg^{-1}). In rats not treated with eugenosedin-A, LPS increased plasma concentrations of NO and prostaglandin E_2 (PGE_2), and levels of p38 MAPK, inducible NO synthase (iNOS) and COX-2 proteins in the liver, lung, aorta and lymphocytes. In the pre-treated rats, eugenosedin-A not only inhibited the LPS-induced NO and PGE_2 levels but also attenuated the LPS-induced increase in p38 MAPK and iNOS levels in the liver, aorta and lymphocytes. Eugenosedin-A also reduced LPS-induced COX-2 proteins in the aorta and lymphocytes. Likewise, aminoguanidine, ascorbic acid, yohimbine and trazodone were also found to decrease NO and PGE_2 concentrations after endotoxin challenge. While aminoguanidine and ascorbic acid also attenuated the LPS-induced increase in p38 MAPK, iNOS and COX-2 proteins in the aorta and lymphocytes, trazodone and yohimbine inhibited only the increase in p38 MAPK, iNOS and COX-2 proteins in lymphocytes. Finally, eugenosedin-A (10^{-10}–10^{-8} M) significantly inhibited the biphasic response induced by hydrogen peroxide (10^{-6}–3×10^{-5} M) in rat denudated aorta. Taken together, the results of this study indicate that eugenosedin-A, as well as ascorbic acid, can attenuate free-radical-mediated aortic contraction and relaxation. It may therefore be able to reduce the damage caused by septic shock by inhibiting formation of p38 MAPK, iNOS, COX-2 and free radicals.

Introduction

Lipopolysaccharide (LPS) and/or cytokines are known to increase levels of inducible nitric oxide synthase (iNOS) in many cell types, including macrophages and vascular smooth muscle cells (Liu et al 2006). The resultant release of large quantities of nitric oxide (NO) has been associated with hypotension and hyporesponsiveness to vasoconstrictor stimuli in endotoxaemia or sepsis (Hollenberg et al 1993). During endotoxaemia, levels of eicosanoids are markedly increased. These lipid mediators are known to contribute to the vascular abnormalities and changes in bronchomotor tone that occur in LPS-induced lung injury (Chang et al 1989; Uhlig et al 1996).

Prostaglandins (PGs), prostacyclin and thromboxane A_2 (TXA_2) are metabolically synthesized from arachidonic acid depending on the activity of cyclooxygenase (COX). Of the 12 PGs, the three most potent are PGD_2, PGE_2 and PGF_2. The other PGs are first-step metabolites in arachidonic acid transformation (PGG_2, PGH_2), products of the degradation of the previously mentioned three PGs, or are not present physiologically, although they may be generated chemically (PGK_2, PGL_2). Two primary COX isoenzymes have been identified: COX-1 (constitutive) and COX-2 (inducible) (Kudo & Murakami 2005). NO and PGE_2 are two inflammatory mediators induced in murine macrophages by the presence

Department of Early Childhood Care and Education, Meiho Institute of Technology, Pingtung, Taiwan
Kuo-Ping Shen

Department of Pharmacy, Chia Nan University of Pharmacy and Science, Tainan, Taiwan
Shu-Fen Liou

Department of Pharmacy, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
Su-Ling Hsieh

Department and Graduate Institute of Pharmacology, Kaohsiung Medical University, Kaohsiung, Taiwan
Ing-Jun Chen, Bin-Nan Wu

Correspondence: Bin-Nan Wu PhD, Department and Graduate Institute of Pharmacology, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 807, Taiwan. E-mail: binnan@kmu.edu.tw

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of LPS (Amin et al 1995) and various combinations of cytokines. Posadas et al (2000) found a co-regulation between iNOS and COX-2, and NO has been found to increase levels of COX-2 (Tetsuka et al 1996). Another study found that NO released from iNOS is capable of activating the inducible COX, resulting in increased production of PGs (Salvenmini et al 1994).

Transcriptional induction of these genes is mediated by intracellular signalling cascades regulated by kinase enzymes. The mitogen-activated protein kinases (MAPKs) are part of such signalling cascades through which diverse extracellular stimuli converge, initiating inflammatory cellular responses. Of the MAPK subgroups, p38 MAPK positively regulates a variety of genes involved in inflammation, including genes for TNF-α, interleukin (IL)-1, IL-6, COX-2 and iNOS (Suh et al 2006).

Reactive oxygen free radicals (such as superoxide anions, hydroxyl radicals and hydrogen peroxide (H_2O_2)) participate in a variety of human diseases, including ischaemia–reperfusion injury and inflammatory diseases (Zweier & Talukder 2006). Sepsis and endotoxaemia also induce the excessive production of free radicals in cells. Free radicals have been implicated in the stimulation of many pro-inflammatory cytokines and mediators involved in acute inflammatory responses associated with sepsis (Blackwell et al 1996). Pro-inflammatory cytokines and mediators are also known to potentiate iNOS and induce COX-2 in endothelial and other cells (Kuo et al 2000; Wu et al 2006). However, antioxidants have been found to scavenge free radicals, decrease levels of iNOS/COX-2 proteins, have an anti-inflammatory effect and may be able to inhibit damage caused by free radicals (Cheng et al 2001; Hsiao et al 2001; Suh et al 2006).

A reciprocally permissive interaction occurs between cytokines and activated α-adrenoceptors. Changes in presynaptic adrenergic sensitivity, as well as in neuronal sensitivity to TNF-α, have been noted in the regulation of norepinephrine associated with certain antidepressant drugs (Nickola et al 2001). This kind of neuro–immune link enables stress-associated norepinephrine to regulate macrophage-derived TNF via α-adrenoceptor interaction. Both norepinephrine and α_2-adrenergic agonists have been shown to augment LPS-induced TNF-α production, though this augmentation can be prevented by yohimbine, an α_2-adrenergic antagonist (Spengler et al 1990). In one of our previous studies using a rat model, we found that when rats were pretreated with eugenosedin-A, an α/β-adrenoceptor and serotonergic receptor blocker (Shen et al 2004), they were protected against hypotension, vascular hyporeactivity, hyperglycaemia, oxidative injury and inflammatory-cytokine formation associated with LPS-induced endotoxaemia. In another of our animal studies, eugenosedin-A was found to more potent than aminoguanidine (NOS inhibitor), ascorbic acid, yohimbine and trazodone in reducing LPS-induced dysfunctions (Shen et al 2005). In this study, we investigated whether eugenosedin-A can prevent endotoxaemia. To do this, we used LPS (10 mg kg^{-1}) to induce endotoxaemia in rats pretreated with eugenosedin-A and measured levels of p38 MAPK, iNOS and COX-2 proteins in various tissues using western blotting and plasma levels of NO and PGE_2. We compare these findings with those from other rats that had been pretreated with other agents (aminoguanidine, ascorbic acid, yohimbine and trazodone) and also subjected to LPS-induced endotoxaemia. In order to characterize the pharmacological properties of eugenosedin-A, we studied its effect on H_2O_2-treated endothelium-denudated rat aorta.

Materials and Methods

Animals

Wistar rats, provided by the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan), were housed under a constant temperature and controlled illumination, with lights on between 7:30 and 19:30. Food and water were available ad libitum. The study was approved by the Animal Care and Use Committee of Kaohsiung Medical University.

Drugs and chemicals

H_2O_2, LPS (*Escherichia coli* serotype 0127:B8) and yohimbine were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Trazodone was obtained from Lotus Medical Supply Co. (Taipei, Taiwan). Eugenosedin-A, 4-{2-hydroxy-3-[1-(2-chlorophenyl-piperazinyl)]-propoxy}-3-methoxy-1-propylenyl-benzene, was synthesized in our laboratory by combining isoeugenol-based oxypropanolamine and 2-chloro-1-piperazinyl benzene (Shen et al 2004). 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 50% absolute alcohol and 10% propylene glycol, and further dilutions were made using distilled water.

In vivo experiment and collection of plasma and tissues

The experiment was performed as described in a previous study (Wu et al 1994). Briefly, Wistar rats weighing 250–300 g were anaesthetized with pentobarbital sodium (50 mg 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, USA) connected to a recorder (model P50; Gould, Valley View, Ohio, USA). Body temperature was maintained at 37°C by an electric heating pad. A femoral vein was cannulated for i.v. injection of test agents and LPS (10 mg kg^{-1}). Eugenosedin-A, yohimbine and trazodone (1 mg kg^{-1}, i.v.), aminoguanidine and ascorbic acid (15 mg kg^{-1} i.v.) were administered 30 min before injection of LPS. Tissues (liver, lung, aorta and lymphocytes) for western blot analysis, and blood samples for the measurement of NO and PGE_2 concentrations in plasma were obtained from rats killed at 1, 3 and 5 h after injection of LPS.

Western blot analysis of p38 MAPK, iNOS and COX-2 protein levels

Tissue samples were obtained from endotoxaemic rats at 1, 3 and 5 h after the injection of LPS and frozen at −70°C before assay. Frozen tissues were homogenized on ice with a polytron

PT 3000 homogenizer (Kinematic AG, Littau, Switzerland) in a buffer composed of (mM): Tris-HCl 50, EDTA 0.1, EGTA 0.1, 2-mercaptoethanol 12 and phenylmethylsulfonyl fluoride 1 (pH 7.4). The homogenized tissues were centrifuged at 10 000 *g* for 30 min and the supernatants were stored at −70°C until further analysis. Aliquots of tissue homogenates were used for protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and western blot analysis. Tissue homogenates containing 20 μg protein were reduced and separated on 7.5% SDS-PAGE gel using PhastSystem with PhastGel (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The membranes were blocked with 1% bovine serum albumin in Tris buffer solution (TBS) containing 0.1% Tween-20 for 2 h and then incubated with anti-rat p38 MAPK, iNOS and COX-2 antibodies (Santa Cruz Biotechnology, CA, USA; 1:500 dilution) in TBS containing 0.1% Tween-20 for 2 h. The membrane was washed and finally incubated with a 1:1000 dilution of anti-mouse immunoglobulin G (Santa Cruz Biotechnology) conjugated to horseradish antibody for 2 h. After successive washings, the immunocomplexes were developed using an enhanced horseradish peroxide/luminol chemiluminescence reaction (ECL western blotting detection reagents, GE Healthcare Bio-Sciences Corp.) and exposed to X-ray film for 10 min. Levels of p38 MAPK, iNOS and COX-2 proteins in each tissue was quantified by densitometric scanning of the western blots using Image-pro plus software (Media Cybernetics, MD, USA) as previously described (Wu et al 2006).

Determination of plasma NO and PGE_2

Blood samples were taken from a catheter placed in the carotid artery, and were centrifuged at 7200 *g* for 10 min. NO and PGE_2 levels in the plasma were measured using a total nitrite colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) and PGE_2 ELISA kit (R&D Systems, Minneapolis, MN) as described previously (Wu et al 2006).

Hydrogen peroxide-induced rat denudated thoracic aorta contraction and relaxation

Thoracic aorta was quickly removed from untreated and pretreated rats, cleaned of adhering fat and connective tissue and cut into 3–4 mm wide transverse rings. The endothelium was removed by gently rubbing the blood vessel against the teeth of a pair of forceps. The rings were then mounted at 1 g resting tension on stainless steel hooks in a 10 mL organ bath, bathed at 37 °C in a 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) for 1 h. The solution was aerated with a 95% oxygen, 5% carbon dioxide. Isometric tension of the aortic rings was monitored by a force displacement transducer (model 7004; Ugo Basile, Italy). H_2O_2 (10^{-6}–3×10^{-5} M) was added to the organ baths containing untreated and pretreated aortic rings to compare their degrees of contraction and relaxation (Wu et al 2001).

Statistical evaluation of data

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 repeated measures or Kruskal–Wallis one-way analysis of variance was used. When this yielded a statistical difference, Dunnett's test or Dunn's test was used to confirm the finding (Jones 2002). A *P* value below 0.05 was considered statistically significant. Analysis of data and plotting of figures was done using SigmaStat: Version 2.03 and SigmaPlot: Version 8.0 (Systat Software, Point Richmond, CA, USA).

Results

p38 MAPK, iNOS and COX-2 protein levels in liver, lung, aorta and lymphocytes after LPS injection

In rats that had not been pretreated with eugenosedin-A, LPS significantly induced the levels of p38 MAPK, iNOS and COX-2 proteins in liver, lung and aorta homogenates by 1, 3 and 5 h. In lymphocytes, LPS challenge markedly increased levels of iNOS at 1, 3 and 5 h; levels of p38 MAPK and COX-2 were increased only at 5 h. Proteins were augmented by LPS in a time-dependent manner, though there was no change in COX-1 protein levels. Surprisingly, none of the three inflammatory proteins could be detected in brain, heart, kidney, pancreas or spleen at any time after LPS challenge (unpublished data).

Pretreatment of rats with eugenosedin-A significantly decreased the effect of LPS stimulation on p38 MAPK levels in liver and aorta (at 1, 3 and 5 h) and lymphocytes (5 h). Eugenosedin-A also reduced iNOS protein in liver (3 and 5 h), aorta and lymphocytes (1, 3 and 5 h) and reduced COX-2 protein in aorta (1, 3 and 5 h) and lymphocytes (5 h). Aminoguanidine decreased LPS-induced p38 MAPK (1 and 3 h), iNOS (1 and 3 h) and COX-2 (1 h) proteins in aorta. It inhibited LPS-induced p38 MAPK (5 h), iNOS (1, 3 and 5 h) and COX-2 (5 h) proteins in lymphocytes. Ascorbic acid decreased LPS-induced p38 MAPK in aorta (1 h) and lymphocyte (5 h), iNOS in aorta (1 and 3 h) and lymphocytes (1, 3 and 5 h), and COX-2 proteins in aorta (1 and 3 h) and lymphocytes (5 h). Trazodone decreased p38 MAPK (5 h), iNOS (1, 3 h) and COX-2 (5 h) proteins in lymphocytes. Yohimbine decreased p38 MAPK (5 h), iNOS (1, 3 and 5 h) and COX-2 (5 h) proteins in lymphocytes. All the agents studied provided greater protection from LPS-induced injury in blood vessels and lymphocytes than in the liver and lungs. Eugenosedin-A in particular reduced LPS-induced inflammatory proteins more potently than aminoguanidine, ascorbic acid, yohimbine and trazodone (Figures 1 and 2, & Table 1). This may explain why previous studies have found eugenosedin-A to be more effective than other test agents in protecting against LPS-induced hypotension and vascular hyporeactivity in rats (Shen et al 2005).

Plasma NO and PGE_2 concentration after LPS injection

Before injecting LPS, there were no significant differences in basal plasma levels of NO and PGE_2 between the experimental

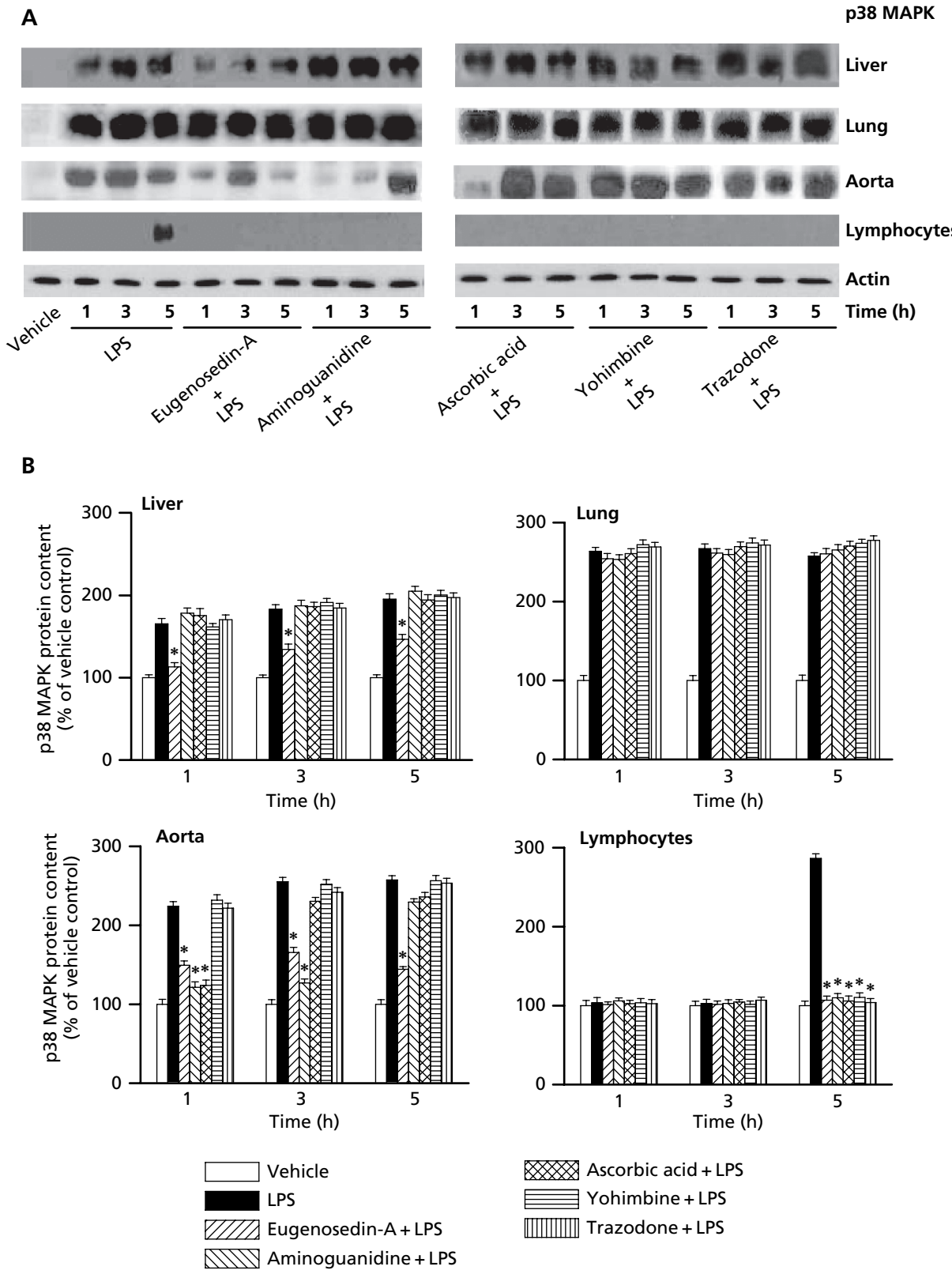

Figure 1 Effects of eugenosedin-A, trazodone, yohimbine (1 mg kg^{-1}, i.v.), aminoguanidine and ascorbic acid (15 mg kg^{-1}, i.v.) on lipopolysaccharide (LPS)-induced p38 MAPK protein levels in rat tissues. Representative western blots of p38 MAPK protein levels in the liver, lung, aorta and lymphocytes (n = 3) (A). Eugenosedin-A and other agents decreased levels of the p38 MAPK protein in various tissues after LPS treatment at 1, 3 and 5 h (B). *$P < 0.05$ compared with the LPS group (Kruskal–Wallis test followed by Dunn's test).

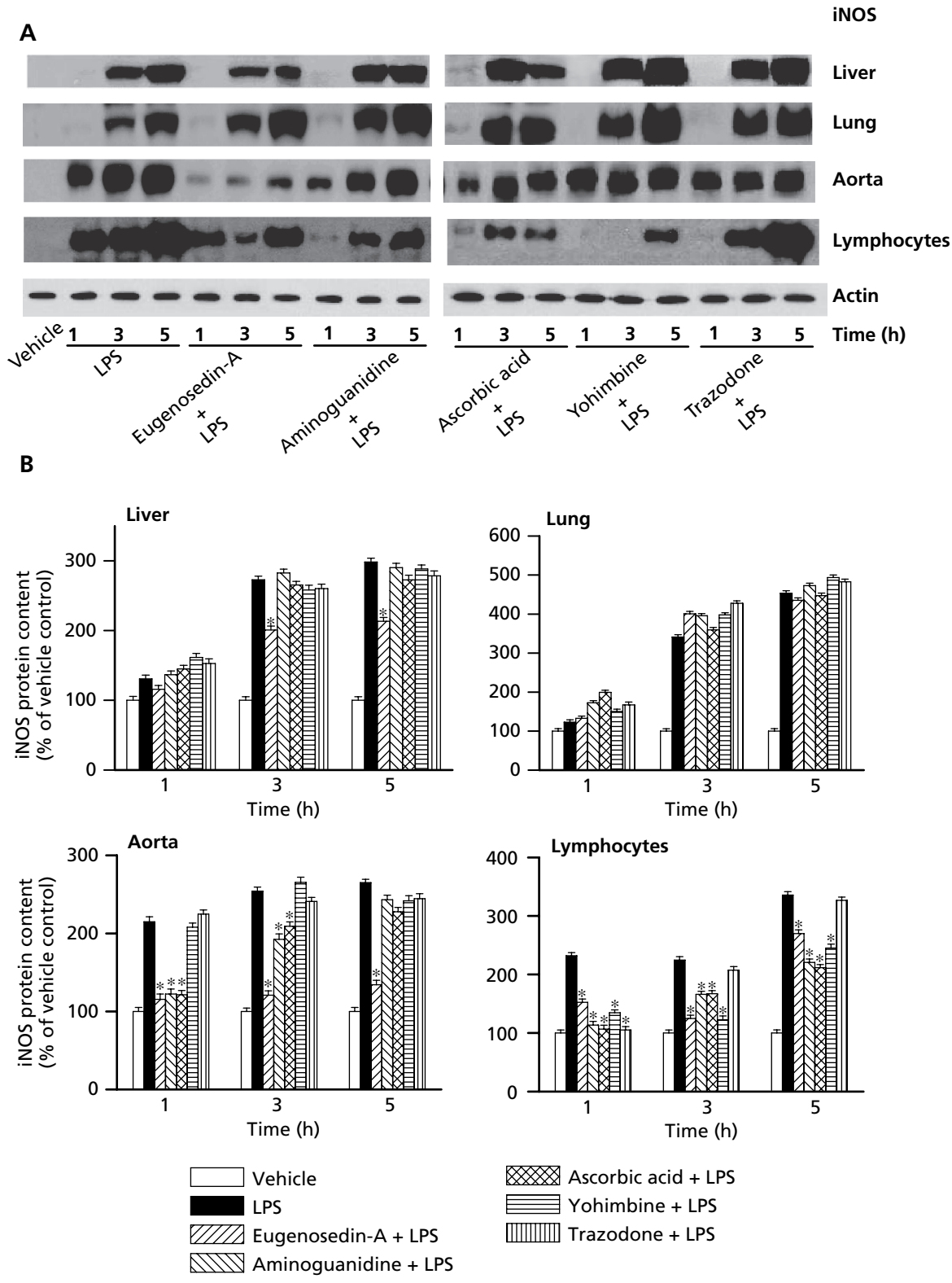

Figure 2 Effects of eugenosedin-A, trazodone, yohimbine (1 mg kg^{-1}, i.v.), aminoguanidine and ascorbic acid (15 mg kg^{-1}, i.v.) on LPS-mediated increase in levels of inducible nitric oxide synthase (iNOS) in rat tissues. Representative western blots of iNOS protein levels in the liver, lung, aorta and lymphocytes (n = 3) (A). Eugenosedin-A and other agents decreased levels of iNOS protein in various tissues after LPS treatment at 1, 3 and 5 h (B). Data indicate the relative optical density of iNOS protein (% of vehicle control). *P <0.05 compared with the LPS group (Kruskal–Wallis test followed by Dunn's test).

Table 1 Effects of eugenosedin-A, trazodone, yohimbine (1 mg kg^{-1}, i.v.), aminoguanidine and ascorbic acid (15 mg kg^{-1}, i.v.) on LPS (10 mg kg^{-1}, i.v.)-induced COX-2 protein product in rat tissues. Eugenosedin-A and other agents decreased the product of COX-2 protein in aorta and lymphocytes after LPS treatment at 1, 3 and 5 h

	Liver			**Lung**			**Aorta**			**Lymphocytes**		
	1 h	3 h	5 h	1 h	3 h	5 h	1 h	3 h	5 h	1 h	3 h	5 h
Vehicle	100.5 ± 3.2	100.1 ± 2.7	100.4 ± 3.5	100.5 ± 5.5	100.4 ± 3.4	100.8 ± 4.6	100.0 ± 0.2	100.0 ± 0.8	100.0 ± 0.7	100.0 ± 0.4	100.0 ± 0.1	100.0 ± 0.7
LPS	146.8 ± 5.6	150.5 ± 6.3	158.6 ± 5.9	200.3 ± 3.9	202.4 ± 6.3	205.9 ± 5.1	269.2 ± 6.7	294.7 ± 6.1	255.2 ± 3.5	100.4 ± 2.2	101.8 ± 3.2	267.6 ± 4.3
Eugenosedin-A + LPS	138.3 ± 3.4	142.3 ± 5.6	154.2 ± 4.1	200.6 ± 5.2	204.3 ± 6.1	208.3 ± 5.1	116.7 ± 5.8*	162.4 ± 5.5*	108.3 ± 6.8*	102.4 ± 3.4	102.6 ± 2.6	101.5 ± 4.1*
Aminoguanidine + LPS	135.6 ± 6.3	152.8 ± 4.1	155.5 ± 5.9	206.7 ± 4.3	204.9 ± 5.6	201.5 ± 5.5	109.1 ± 6.6*	286.9 ± 4.9	272.6 ± 6.8	100.5 ± 4.5	103.1 ± 3.8	103.5 ± 4.1*
Ascorbic acid + LPS	144.3 ± 5.6	149.6 ± 5.8	160.7 ± 6.9	202.8 ± 5.9	206.4 ± 5.2	205.7 ± 5.7	100.1 ± 5.3*	100.8 ± 3.4*	272.8 ± 6.2	101.3 ± 5.1	103.4 ± 4.8	103.1 ± 3.7*
Yohimbine + LPS	155.8 ± 5.3	163.3 ± 5.7	166.4 ± 5.3	202.7 ± 5.2	205.7 ± 6.2	202.4 ± 6.3	277.5 ± 6.4	277.1 ± 5.3	264.8 ± 6.3	104.2 ± 3.2	102.3 ± 3.4	104.5 ± 5.8*
Trazodone + LPS	159.6 ± 6.3	157.1 ± 6.6	161.8 ± 4.8	205.5 ± 5.4	203.3 ± 4.6	206.4 ± 5.3	260.2 ± 7.2	284.1 ± 5.1	252.2 ± 5.3	100.5 ± 4.6	103.7 ± 4.1	102.8 ± 4.1*

Data indicate the relative optical density of COX-2 protein (% of vehicle control).
*Significantly different from the LPS group, $P < 0.05$ (n = 3) (Kruskal–Wallis one-way analysis of variance followed by Dunn's test).

groups. Injection of LPS significantly increased plasma levels of NO and PGE2 in a time-dependent manner. Pretreatment of rats with eugenosedin-A, trazodone, yohimbine, aminoguanidine or ascorbic acid clearly inhibited the LPS-induced increases in NO and PGE_2 levels in plasma (Table 2).

Inhibition by eugenosedin-A of H_2O_2-induced endothelium-independent contraction and relaxation in the isolated aorta

H_2O_2 (10^{-6}–3×10^{-5} M) elicited biphasic effects in rat denudated thoracic aorta from untreated rats. The rat aorta contracted at lower concentrations of H_2O_2 but this contraction decreased sharply at higher concentrations of H_2O_2 (approximately 5×10^{-4} M) (Figure 3). This biphasic effect of H_2O_2 was greatly inhibited in rats pre-treated with eugenosedin-A or ascorbic acid. Aminoguanidine, yohimbine and trazodone, however, did not attenuate the responses of denudated rat aortic rings to H_2O_2.

Discussion

In our previous report (Shen et al 2004), eugenosedin-A was shown to possess α_1/α_2-adrenoceptor and β_1- (but not β_2) adrenoceptor antagonistic activities, and to have serotonergic receptor blocking actions. In another of our studies (Shen et al 2005), eugenosedin-A was found to have antioxidant effects, scavenged free radicals and prevented LPS-induced hypotension, vascular hyporeactivity, hyperglycaemia, oxidative injury and formation of inflammatory cytokines in rats. Here we investigated its effect on NO and PGE_2 production and

Table 2 Effect of eugenosedin-A, yohimbine, trazodone (1 mg kg^{-1}), aminoguanidine and ascorbic acid (15 mg kg^{-1}) on lipopolysaccharide (LPS)-induced changes of nitric oxide (NO) and prostaglandin (PGE_2) levels in plasma at 1, 3 and 5 h

Treatment	NO (μM)			PGE_2 (ng mL^{-1})		
	1 h	3 h	5 h	1 h	3 h	5 h
Vehicle	3.0 ± 1.5	3.1 ± 1.1	3.1 ± 1.0	2.5 ± 1.6	2.5 ± 1.5	2.5 ± 1.5
Vehicle + LPS	15.1 ± 1.2	32.6 ± 1.7	215.6 ± 5.5	11.5 ± 1.2	12.0 ± 1.1	12.1 ± 1.1
Eugenosedin-A + LPS	7.2 ± 1.5*	19.6 ± 1.9*	140.9 ± 9.1*	4.6 ± 1.3*	7.5 ± 1.2*	5.7 ± 1.2*
Aminoguanidine + LPS	8.5 ± 1.3*	18.1 ± 2.4*	150.5 ± 10.4*	3.4 ± 1.2*	7.8 ± 1.5*	5.3 ± 1.1*
Ascorbic acid + LPS	9.1 ± 1.2*	20.6 ± 1.7*	148.4 ± 13.9*	5.7 ± 1.5*	8.1 ± 1.2*	6.7 ± 1.2*
Yohimbine + LPS	8.2 ± 1.4*	19.5 ± 1.5*	160.4 ± 11.4*	5.3 ± 1.2*	7.4 ± 1.1*	6.3 ± 1.1*
Trazodone + LPS	9.0 ± 1.4*	17.4 ± 2.2*	198.6 ± 15.9*	7.0 ± 1.3*	7.3 ± 1.0*	7.4 ± 1.1*

Values are the mean ± s.e., n = 8 rats.
*$P < 0.05$ compared with vehicle + LPS, (one-way repeated measures analysis of variance, followed by Dunnett's test).

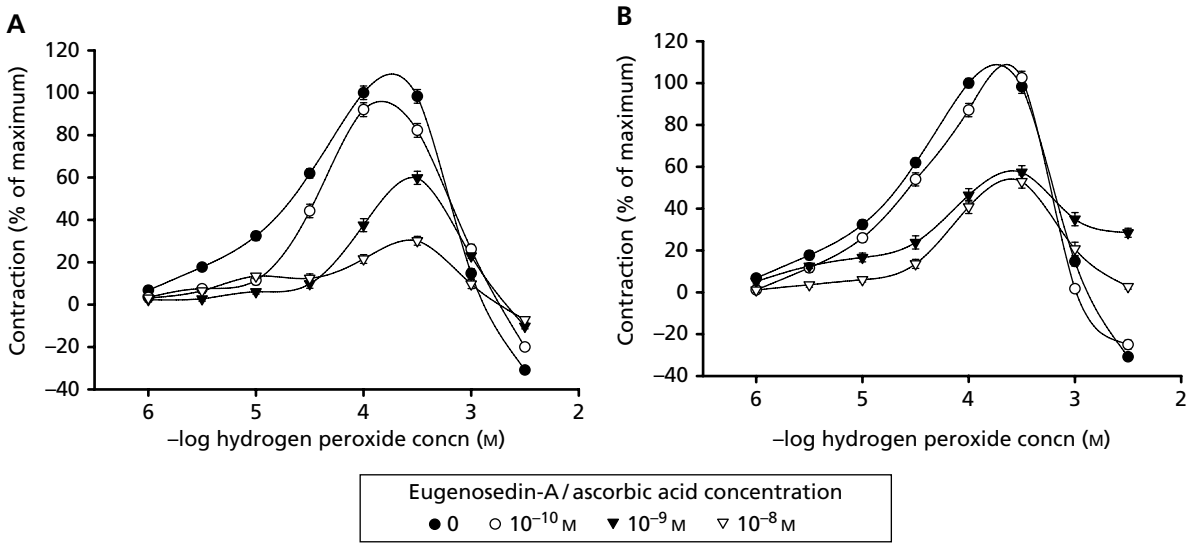

Figure 3 Antagonism of hydrogen-peroxide-induced vasocontraction and vasorelaxation in the absence and presence of eugenosedin-A (A) or ascorbic acid (B). Cumulative concentration–response curves were determined in endothelium-denuded thoracic aortic rings prepared from Wistar rats. Each value represents the mean ± s.e., n = 8.

levels of p38 MAPK, iNOS and COX-2 proteins with LPS-induced endotoxaemia. Eugenosedin-A was found to scavenge free radicals in H_2O_2-induced endothelium-independent contraction and relaxation in the aorta.

LPS-induced activation of *p38 MAPK* is thought to correspond to the LPS-positive regulation of a variety of genes involved in inflammation in-vitro and in-vivo (Ono & Han 2000). *p38 MAPK* signalling has been implicated in the activation of activator protein-1 (AP-1) by LPS, which binds to the AP-1 site in the *iNOS* and *COX-2* promoter, as was also observed in *iNOS* and *COX-2* transcription (Kristof et al 2001). Therefore, *p38 MAPK* signalling may form the basis of a new strategy for treating inflammatory diseases (Ono & Han 2000). In fact, several potent p38 MAPK inhibitors have been developed and evaluated in animal models of inflammatory diseases (Jeon et al 2000; Nick et al 2000), and p38 MAPK inhibitors have been found to have anti-inflammatory effects in human endotoxaemia (Branger et al 2002; Suh et al 2006). In the present study, eugenosedin-A reduced levels of p38 MAPK protein after LPS challenge. It also inhibited both iNOS and COX-2 proteins. These results indicate that eugenosedin-A plays an important role in inhibiting the stimulation of *p38 MAPK* that occurs during endotoxaemia, and may potentially be used in the treatment of inflammatory diseases. Endotoxaemia causes the release of a number of mediators, including cytokines, proteinase, NO, eicosanoids and toxic oxygen metabolites. LPS and various cytokines have been found to stimulate the production of iNOS, which may contribute to the pathological consequences of shock, and they increase the production of NO in plasma (Hsiao et al 2003). In our study, the introduction of LPS increased levels of iNOS in rat liver, lung, aorta and lymphocytes. When rats were pre-treated with eugenosedin-A before being injected with LPS, levels of iNOS were significantly lower in the liver, aorta and lymphocytes. With regard to this measure in rats pre-treated with other agents before endotoxaemia was induced, aminoguanidine and ascorbic acid reduced iNOS levels in aorta and lymphocytes, and yohimbine and trazodone reduced iNOS levels only in lymphocytes. All agents were found to inhibit production of NO in plasma after LPS injection. One of our previous studies reported that eugenosedin-A, trazodone, yohimbine, aminoguanidine and ascorbic acid could normalize LPS-induced hypotension and vascular hyporeactivity (Shen et al 2005). One study has reported that the presence of α_2-adrenoceptor antagonists in mice brought about a significant decrease in LPS-induced TNF-α plasma levels (Spengler et al 1990), and another study has reported that various aspects of the inflammatory response could be suppressed by 5-hydroxytryptamine (5-HT; serotonin), 5-HT_{1A}, 5-$HT_{2A/2C}$ receptor antagonists, selective serotonin reuptake inhibitors and depletion of intracellular 5-HT (Smejkal-Jagar & Boranic 1994). In the current study, we found a clear relationship between the levels of iNOS protein and p38 MAPK protein in the liver, lung and aorta after LPS injection, but there was not such a clear relationship between the two in lymphocytes. Future studies will be needed to determine whether *MAPK* signalling is associated with increased iNOS activity in lymphocytes.

This study found that eugenosedin-A, yohimbine and trazodone reduced the levels of iNOS protein and NO in rats after the injection of LPS, suggesting that α_2-adrenoceptor antagonists and 5-HT antagonists may be able to improve LPS-induced dysfunction relating to the inhibition of iNOS activity. By inhibiting iNOS product, some antioxidants have been found to preserve the activity of constitutive NOS (Cheng et al 2001; Hsaio et al 2001). Other studies have suggested that ascorbate may prevent direct injury by oxidants associated with sepsis (Wu et al 2004) and that some antioxidants may be used to selectively inhibit the induction of iNOS during sepsis (Wu et al 2002). Taken together, these observations suggest that eugenosedin-A has similar effects to those of α_2-antagonists, 5-HT antagonists and antioxidants in inhibiting LPS-induced iNOS. Hyperglycaemia in the early phase of sepsis has been reported to be caused by a decrease in peripheral tissue glucose uptake relative to the rate of glucose production, and iNOS has been proposed to contribute to the development of LPS-induced hyperglycaemia in signalling mechanisms (Gupta et al 2004). Our study demonstrated that eugenosedin-A, aminoguanidine and ascorbic acid were more potent inhibitors of *iNOS* product than yohimbine and trazodone. These findings may explain why eugenosedin-A, aminoguanidine and ascorbic acid can significantly attenuate LPS-induced hyperglycaemia and why yohimbine and trazodone cannot (Shen et al 2005).

Prostanoids regulate a number of physiopathological processes and are synthesized by two COX isoforms. COX-1 appears to synthesize the prostanoids involved in homeostatic functions, whereas COX-2, which is present in low levels under basal conditions, is up-regulated by inflammatory stimuli, resulting in increased PG production (Ristimäki et al 1994). There is the possibility of an interaction between NO and COX. It has been reported that, in vitro, NO potentiates cytokine-induced PGE_2 production in a number of cell systems, including vascular smooth muscle cells (Inoue et al 1993), rat mesangial cells (Tetsuka et al 1994), human microglial cells (Janabi et al 1996) and human airway epithelial cells (Watkins et al 1997). NO may indirectly affect the production of PGE_2 by stimulating an increase in COX-2 protein levels (Tetsuka et al 1996). NO has been found to play a regulatory role in the production of prostanoids in-vivo in rats treated with LPS (Salvemini et al 1995). In a model of renal inflammation in rabbits, NO released from iNOS activated the induction of COX, resulting in increased production of PGs (Salvemini et al 1994). Egger et al (2003) have reported that α-tocopherol, an antioxidant, inhibited the LPS-activated NF-κB signalling cascade, resulting in a significant attenuation of COX-2 protein synthesis. In the present study, we found that levels of COX-2 were related to p38 MAPK in lung, aorta and lymphocytes, but not in liver. Given the time course of these effects, there may have not been enough time for p38 MAPK to stimulate the changes of *COX-2* product in liver. Eugenosedin-A, aminoguanidine and ascorbic acid decreased COX-2 protein in aorta and lymphocytes in rats injected with LPS. Yohimbine and trazodone diminished COX-2 levels only in lymphocytes. All the agents we studied reduced LPS-induced COX-2 to a greater extent in blood vessels and lymphocytes than in the liver or lung. Eugenosedin-A, aminoguanidine and ascorbic acid had more potent effects in inhibiting the product of COX-2 than yohimbine and trazodone. All agents reduced increases in plasma LPS-induced

PGE_2 levels. These findings have led us to hypothesize that α_2-antagonists, 5-HT antagonists, iNOS inhibitors and antioxidants may be able to prevent LPS-induced induction of COX-2.

Several reports have shown that the effects of catecholamines on immune function is mediated via β-adrenoceptors. The stimulation of iNOS synthesis by epinephrine and norepinephrine on LPS-induced macrophages was down regulated by β-adrenoceptor antagonists (Chi et al 2003). Rauwolscine, an α_2-adrenoceptor blocker, inhibited the tissue dysfunction after LPS injection (Fessler et al 1996). Therefore, α_2-adrenoceptor activation may be involved in LPS-mediated injury, and α_2-adrenoceptor antagonists protect against LPS, either by decreasing TNF-α production or through direct effects on the target tissues of endotoxaemia (Kazerani and Furman, 2006). On the other hand, 5-HT has been shown to induce the early-response gene *COX-2*. Of the multiple 5-HT receptors, the 5-HT_{2A} subtype was shown to be responsible for the mitogenic response and induction of *COX-2* (Goppelt-Struebe & Stroebel, 1998). In this study, we suggested that eugenosedin-A attenuates LPS-induced iNOS and COX-2 protein formation, relating to its α/β-adrenoceptor antagonist and serotonergic receptor antagonist activities (Figure 4). Elucidation of the detailed signalling pathway from cytoplasm to nucleus is our next challenge.

H_2O_2, a reactive oxygen species, is an important by-product of oxidative metabolism. Excessive amounts of reactive oxygen species can be induced by sepsis and endotoxaemia. H_2O_2, which can be formed by the vascular endothelium and smooth muscle cells (Gutteridge 1995), can produce concentration-dependent contraction in isolated endothelium-denuded rat aorta rings (Yang et al 1998), although it has also been reported to relax endothelium-intact rabbit aorta and norepinephrine-precontracted aorta in a concentration-dependent manner (Bharadwaj & Prasad 1997). H_2O_2 contraction of the aorta is stimulated by the influx of calcium ions, which can be increased by the production of PGD_2, $PGF_2\alpha$ and TXA_2 (Yang et al 1998). Relaxation of aortic tissues by H_2O_2 is mediated through release of an endothelium-derived relaxing factor; this relaxation is absent from aorta denuded of endothelium (Bharadwaj & Prasad 1997). Whether H_2O_2 produces direct relaxation of vascular smooth muscle via stimulation of guanylate cyclase (Mian & Martin 1995) or via potassium-channel opening (Matoba et al 2000) has not been decided.

In the present study, lower concentrations of H_2O_2 induced contractions in denudated thoracic rat aorta, whereas higher concentrations induced relaxation. Eugenosedin-A and ascorbic acid significantly inhibited H_2O_2-induced biphasic effects. Previously, eugenosedin-A was shown to possess free-radical-scavenging properties and inhibited lipid peroxidation (Shen et al 2005). Thus, we suggest that eugenosedin-A and ascorbic acid inhibit H_2O_2-induced contractions and relaxation in rat denudated thoracic aorta by scavenging free radicals.

We have previously reported that eugenosedin-A attenuated LPS-induced cardiovascular dysfunction, hyperglycaemia, oxidative injury and inflammatory cytokine formation (Shen et al 2005). The present study shows that eugenosedin-A can potently prevent LPS-induced p38 MAPK, iNOS and COX-2 enhancement in various tissues. Furthermore, we found that eugenosedin-A could inhibit LPS-induced increases in NO and PGE_2 and that it was able to decrease free radicals. This evidence explains why eugenosedin-A is a more effective inhibitor of LPS-induced cardiovascular dysfunction, hyperglycaemia, oxidative injury and inflammatory cytokine formation than aminoguanidine, ascorbic acid, yohimbine or trazodone. Together, these findings suggest that the protective effect of eugenosedin-A against LPS-induced dysfunction may be related to diminished release of cytokines and production of p38 MAPK, iNOS and COX-2 proteins and reduced accumulation of free radicals.

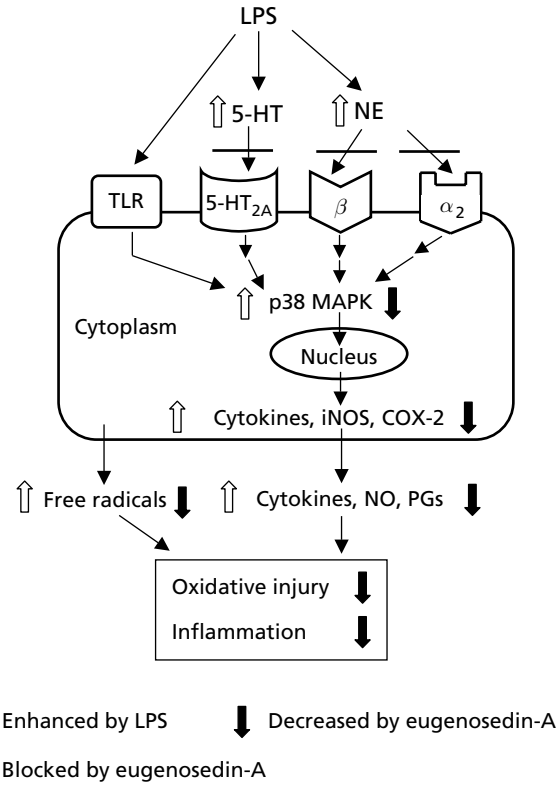

Figure 4 Proposed mechanisms of action of eugenosedin-A on lipopolysaccharide (LPS)-induced oxidative injury and inflammation. LPS modulation of cytokines, nitric oxide (NO) and prostaglandin (PG) formation may directly activate Toll-like receptors (TOLR) and indirectly stimulate endogenous 5-hydroxytryptamine (5-HT; serotonin) and norepinephrine (NE) release, resulting in the increasing of p38 mitogen-activated protein kinase (*p38 MAPK*) product. Eugenosedin-A blocks α- and β-adrenoceptors and 5-HT receptors to attenuate LPS-induced formation of cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Eugenosedin-A diminishes accumulation of free radicals, which also protects against LPS-induced oxidative injury.

Conclusion

This study demonstrated that eugenosedin-A, an α/β-adrenoceptor and serotonergic receptor blocker, is effective in protecting rats against LPS-induced systemic inflammatory responses. In light of these results, we suggest that the anti-inflammatory

mechanisms of eugenosedin-A might result from the inhibition of iNOS and COX-2 through reductions in p38 MAPK. Eugenosedin-A could potentially be used as a preventive or even as a novel therapeutic agent in endotoxaemia and associated septic shock.

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