Effects of endothelin receptor antagonists on endothelin-1 and inducible nitric oxide synthase genes in a rat endotoxic shock model

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ABSTRACT

Levels of the endothelium-derived vasoconstrictor endothelin (ET)-1 and the vasodilator nitric oxide (NO) are markedly increased in endotoxic shock, although the pathophysiological role of ET-1 and its relation to NO under septic conditions remains obscure. To delineate the roles of ET-1 and the ET receptors, and the NO/inducible NO synthase (iNOS) system in endotoxic shock, we examined the gene expression of ET-1, ET receptors A and B (ETA and ETB) and iNOS in the heart and the liver of a rat endotoxic shock model, and we studied the effects of ET receptor antagonists on haemodynamics, survival rate and expression of ET-1, ET receptors and iNOS. Administration of bacterial lipopolysaccharide (LPS) into rats caused a profound hypotension with resultant death. However, these effects were blocked by a non-selective ETA/ETB receptor antagonist (TAK044), but not by an ETA-selective antagonist (BQ123). Injection of LPS caused a marked elevation in the plasma levels of both ET-1 and NO, which were not affected by treatment with either ET receptor antagonist. Administration of LPS caused increases in levels of ET-1, ETB and iNOS mRNA in the heart and the liver, whereas ETA mRNA expression was markedly downregulated in these organs. These results suggest that ET receptor subtype genes are differentially regulated in major organs from endotoxic shock rats, and that non-selective ET receptor antagonists ameliorate endotoxin-induced hypotension and death irrespective of iNOS-derived NO.

INTRODUCTION

Endothelium-derived vasoactive factors play important roles in the maintenance of vascular tonus and regional blood flow. Among them, endothelin-1 (ET-1), a potent vasoconstrictor peptide, and nitric oxide (NO), a potent vasodilator, are regarded as key molecules to counteract each other [1]. There is a close interaction between ET-1 and NO: ET-1 stimulates NO release by activation of endothelial NO synthase via the ETB receptor, while NO inhibits ET-1 release by blocking ET-1 gene expression.

In septic shock, circulating levels of both ET-1 and NO have been shown to be markedly elevated. Excessive production of NO by inducible NO synthase (iNOS) has been considered to be largely responsible for the development of a profound and intractable hypotension, a hallmark of endotoxic shock. Bacterial lipopolysaccharide (LPS) and several proinflammatory cytokines, such as interleukin-1 and tumour necrosis factor α, induce iNOS gene expression in a variety of cells, while LPS and these cytokines have also been shown to induce ET-1 gene expression in endothelial cells. However, it remains controversial as to whether enhanced ET-1 expression may contribute to the maintenance of blood pressure and/or the development of multiple organ failure under endotoxic shock state.

Recently, several ET receptor antagonists have been
developed and used for the treatment of various cardiovascular diseases including septic shock [2]. However, these receptor antagonists are either for ET-1-selective ETA receptors (e.g. BQ123), or for non-selective ETA/ETB receptors (e.g. bosentan and TAK044). BQ123 has been shown to attenuate ET-1-induced pulmonary vasoconstriction in rat endotoxic shock [3]. Bosentan, on the other hand, has been shown to ameliorate pulmonary hypertension and increase cardiac output and regional blood flow in porcine endotoxic shock [4]. These results suggest the possible involvement of both ETA and ETB receptors in the maintenance of blood pressure under endotoxic shock conditions.

To delineate the roles of both ET-1/ET receptors and the NO/iNOS system in the endotoxic shock state, the present study determined the expression of ET-1, ETA, ETB and iNOS genes in the heart and liver of rat endotoxic shock model, and studied the effects of two ET receptor antagonists (BQ123 and TAK044) on haemodynamics, survival rate and circulating ET-1 and NO levels as well as tissue expression of ET-1/ET receptors and iNOS genes in endotoxic shock rats.

**MATERIALS AND METHODS**

**Measurement of haemodynamic changes**

The experimental procedures were in accordance with the institutional guidelines for animal studies. Male Wistar rats (300–350 g) were anaesthetized and continuous recordings of mean arterial pressure (MAP) and heart rate were recorded via the femoral artery using a pressure transducer. After a 30-min equilibrium period, animals received an intravenous administration of BQ123 (30 mg/kg of body weight), TAK044 (1 mg/kg of body weight) or vehicle (saline) as a bolus, and 30 min later, a 10-min continuous infusion of LPS (20 mg/kg of body weight) or vehicle (saline). Blood was withdrawn and animals were sacrificed 5 h after injection of LPS or vehicle. The heart and the liver were removed and immediately frozen in liquid nitrogen. Tissue samples were stored at −80 °C until analysis.

**Measurement of plasma ET-1 and NO**

After extraction with Sep-Pak C-18 columns, plasma ET-1 level was determined by radioimmunoassay using an ET-1 assay kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Plasma NO level was determined by chemiluminescence using an NO analyser (Sievers, Boulder, CO, U.S.A.).

**Quantification of ET-1, ETA and ETB receptor, and iNOS mRNAs**

cDNA was synthesized from total RNA extracted from the tissues with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech) using the random primer, pd(N)6. Real-time quantitative PCR was performed using a LightCycler™ (Roche Boehringer, Mannheim, Germany) [5]. The sequences of primers for rat ET-1, ETA and ETB receptors and iNOS were as follows: ET-1, (forward) 5′-CGTCCGTATGGACTAGGAA-3′ and (reverse) 5′-TGCAACTGAGGAGGCTC-3′; ETA, (forward) 5′-ATCGGGATCCCTCCTGATTAC-3′ and (reverse) 5′-TGCAAACCAAGCGAGACGGAG-3′; ETB, (forward) 5′-GGACTACAAGGGAAAAGCC-3′ and (reverse) 5′-TGTCACCAGATACACAGGCG-3′; and iNOS, (forward) 5′-GAAGGCTGGAACTAAGGC-3′ and (reverse) 5′-GTGCTGTGCTGAATGACCCGG-3′. cDNA template was mixed with reaction mixture containing 4 µM MgCl₂ and 0.5 µM primers. Amplification temperature profiles were optimized for each set of primers: 95 °C for 1 min followed by 40 cycles of 95 °C for 1 s, 60 °C for 5 s and 72 °C for 20 s [except for the annealing temperatures for ETA (57 °C) and ETB (54 °C)]. The temperature ramp rate was 20 °C/s. After the completion of each extension step at 72 °C, the fluorescence of each sample was measured at 82 °C. After amplification, the products were subjected to a temperature gradient from 65 °C to 95 °C at 0.2 °C/s with continuous fluorescence monitoring; the fluorescence was quantitatively analysed using standard controls, allowing quantification of templates.

**RESULTS**

**Effects of ET receptor antagonists on MAP and death**

Injection of LPS caused a marked decrease in MAP from 91.2 ± 5.7 mmHg to 48.5 ± 3.2 mmHg after 5 h. In the BQ123-pretreated group, administration of LPS caused an earlier decrease in MAP; the lowest value, 58.0 ± 7.2 mmHg, was observed after 4 h. In contrast, pretreatment with TAK044 prevented the LPS-induced decrease in MAP after 5 h.

In the LPS group, the mortality rate was 33.3% at 5 h. TAK044 prevented LPS-induced death and all rats survived after 5 h. In contrast, BQ123 increased the mortality rate and all rats died before 4 h.

**Changes of circulating ET-1 and NO**

After LPS administration, plasma concentrations of ET-1 and NO increased significantly compared with those seen in vehicle-treated rats; these changes were unaffected by pretreatment with BQ123 or TAK044 (Figure 1).

**Expression ET-1, iNOS and ET receptor mRNAs in major organs**

Steady-state mRNA levels of both ET-1 and iNOS in the heart and the liver increased significantly after admini-
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Figure 1 Changes of plasma levels of ET-1 (left) and NO (right) in endotoxic shock rats
Rats were pretreated with ET receptor antagonists or were left untreated. White bar, vehicle and no antagonists; black bar, LPS and no antagonist; diagonal shading, BQ123 and LPS; horizontal shading, TAK044 and LPS.

Figure 2 Changes of ET-1 and iNOS mRNA levels in the heart and the liver from endotoxic shock rats
Rats were pretreated with or without ET receptor antagonists. White bar, vehicle and no antagonists; black bar, LPS and no antagonist; diagonal shading, BQ123 and LPS, horizontal shading; TAK044 and LPS.

This study shows clearly that administration of bacterial LPS to rats caused a profound hypotension associated with marked increases in plasma ET-1 and NO levels. These results are in agreement with those of previous studies in endotoxic shock patients [6] and several endotoxic shock animal models [7,8].

Using a quantitative reverse transcription-PCR, the present study further reveals that steady-state mRNA levels of both ET-1 and iNOS were enhanced in the heart and the lung from endotoxic shock rats. These results corroborate the results that showed increased circulating ET-1 and NO levels in endotoxic shock rats. Therefore, it is likely that enhanced secretion of ET-1 and NO is derived from upregulated ET-1 and iNOS genes in the affected organs during endotoxaemia. Since LPS and proinflammatory cytokines (interleukin-1, tumour necrosis factor α) induce ET-1 and iNOS genes in vascular endothelium and smooth muscle respectively, these factors that are increased during endotoxaemia are major...
candidates for the upregulation of ET-1 and iNOS genes in these tissues.

It has been shown that ETA receptor mRNA expression was markedly downregulated in the major organs from endotoxic shock rats, whereas ETB receptor mRNA levels increased, suggesting differential regulation of ET receptor subtype genes under endotoxic shock state. The mechanism(s) by which ET receptor subtypes are differentially regulated remains obscure. However, it is possible that expression of ET receptor subtypes may be modulated by several hormones and cytokines associated with endotoxaemia [1]. For example, ETA expression may be downregulated by ET-1 itself, glucocorticoid, platelet-derived growth factor and transforming growth factor β. Conversely, ETB expression may be upregulated by catecholamines and angiotensin, both of whose levels have been shown to be markedly elevated during sepsis. This differential regulation of ET receptor subtypes may be a compensatory mechanism for damping the ET-1-mediated potent vasoconstriction in response to endotoxic shock.

It has been shown that TAK044, a non-selective ETA/ETB receptor antagonist, but not BQ123, a selective ETA receptor antagonist, improved pulmonary pressure and increased survival of endotoxic shock rats. These results are comparable with those of previous studies. It has been reported that an ET receptor antagonist (SB209670) augmented the degree of hypotension, vascular hyporeactivity to noradrenaline, renal dysfunction and metabolic acidosis in endotoxic shock rats [9]. In contrast, it has been reported that a non-selective ET receptor antagonist (bosentan) abolished pulmonary hypertension, improved cardiac output and increased visceral blood flow without causing a further decrease in MAP in endotoxic shock pigs [4]. Furthermore, we have recently shown that the non-selective ET receptor antagonist (TAK044) prevented metabolic acidosis and hypoxaemia, and improved renal dysfunction without causing a further decrease in MAP in a canine endotoxic shock model [10].

It should be noted that activation of ETB receptor causes vasoconstriction in certain vascular smooth muscles [11,12]. Therefore, it is possible that blockade of ETB receptor-mediated vasoconstriction may lead to improvement of decreased regional blood flow. However, it has been reported that a selective ETB receptor antagonist (RES-701-1) increased cardiac pressure and decreased cardiac output in a canine heart failure model [13]. The question of whether a selective ETB receptor antagonist is therapeutically better than a non-selective ETA/ETB receptor antagonist for treatment of endotoxic shock remains to be answered.

Finally, the present study shows that increased circulating NO levels, as well as augmented iNOS expression in major organs from endotoxic shock rats, were not affected by either selective blockade of ETA receptor or non-selective blockade of ETA/ETB receptors. These data exclude the involvement of endogenous ET-1 in the mechanism of iNOS induction and subsequent excessive NO production during endotoxaemia. Raised plasma ET-1 levels were almost comparable between BQ123- and TAK044-treated animals. Since blockade of ETB receptor impairs pulmonary clearance of endogenous ET-1 and increases plasma ET-1 levels [14], concomitant blockade of ETA and ETB receptors by TAK044 may only marginally affect the clearance of ET-1.

**REFERENCES**