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Endothelin receptor antagonist bosentan improves survival in a murine caecal ligation and puncture model of septic shock

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Abstract

The role of endothelin peptides was evaluated on survival and organ injury in a model of polymicrobial sepsis, induced by caecal ligation and puncture with particular emphasis on the timing of the administration of its blocker bosentan in Swiss albino mice (20–40 g). The cardiovascular response pattern in this experimental model was characterized by an early, "hyperdynamic" phase starting at 5 h, followed by a late but "hypodynamic" phase that commence after 20 h, provided that the animals are "resuscitated" by injecting 1 ml of saline i.p. at the end of the surgery. However, if saline resuscitation is omitted, then only hypodynamic pattern is observed starting at 5 h without any hyperdynamic phase. Thus, mice were first allocated into saline-resuscitated or unresuscitated groups and endothelin receptor antagonist bosentan (30 mg kg^{-1}, i.p., either 5 or 20 h after caecal ligation and puncture) was then administered. The control animals received the solvent of bosentan (i.e., saline: %0.9 NaCl, w/v). The survival rates in each group (n=14) were recorded over the following 144 h. In unresuscitated mice, the overall survival at 144 h was 14.3% in controls while bosentan treatment at 5 h (78.6%, P=0.0018) or 20 h (64.3%, P=0.0183) have both significantly improved the survival. However, in saline-resuscitated mice, bosentan administered at 20 h has significantly improved the survival (71.4%, P=0.0213) while its administration at 5 h has yielded exactly the same percent of survival (i.e., 21.4%) as observed in control animals. The beneficial effects of bosentan in preventing the tissue injury due to caecal ligation and puncture were also observed histopathologically in liver, spleen and kidney. Therefore, we concluded that the blockade of endothelin receptors by using bosentan during the later (hypodynamic) stages of septic shock is a promising therapeutic manoeuvre.

Keywords: Septic shock; Endothelin; Bosentan; Organ injury; Survival

1. Introduction

Sepsis is a heterogeneous class of syndromes caused by a systemic inflammatory response to infection. Septic shock, a severe form of sepsis, is associated with the development of progressive damage in multiple organs, and is the leading cause of patient mortality in intensive care units (Bone, 1991). Despite advances in the management of the septic patient, as well as the understanding of pathophysiological mechanisms of sepsis, the high mortality rate and the ensuing multiple organ failure have not been significantly reduced in the past two decades (Rangel-Frausto et al., 1995).

Although there exists an extensive amount of studies which demonstrated significant beneficial effects of various procedures in experimental animals, there is controversy on their validity with regard to their relevance to human sepsis. Principally, the studies are focused on demonstrating the beneficial effects of prophylactic measures taken "before" the initiation of sepsis rather than the usefulness of therapeutic interventions that are taken "after" the development of sepsis. Likewise, the pitfalls of various models which utilize endotoxin to mimic the human clinical situation are also widely criticised (Villa et al., 1995). In particular, the shortcomings of these models in realistically reflecting complex immune response and cytokines appear

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to be the major problem (Villa et al., 1995). In contrast, caecal ligation and puncture model is becoming regarded as superior in reflecting various facets of the systemic response to local infection (Chaudry et al., 1979; Wichterman et al., 1980). It is similar to the clinical situation of bowel perforation which induces peritonitis due to mixed intestinal flora. In this model of murine sepsis, if the animals were injected 1 ml of normal saline immediately after the surgery, they exhibit an early "hyperdynamic" stage of sepsis at 5 h and they progress to a late "hypodynamic" stage 20 h after caecal ligation and puncture (Wang and Chaudry, 1996; Yang et al., 2002). The validity of caecal ligation and puncture has also been supported by publications that showed that the blockade of tumour necrosis factor alpha has failed to prevent death in this model (Remick et al., 1995) which is similar to that observed in various human clinical trials.

In our quest to better understand the underlying mechanisms of septic shock, we have previously reported that it was possible to improve the survival by blocking the endothelin receptors by using bosentan, a dual (ET_A–ET_B) endothelin receptor antagonist with no intrinsic agonist activity (Clozel et al., 1994), during the late "hypodynamic" stage of sepsis in a mice model of *Escherichia coli* endotoxin (Lipopolysaccharide O55:B5)-induced septic shock with a rather high mortality rate of 90% at 24 h (Iskit and Guc, 2000, 2001). Moreover, we have also denoted that bosentan was beneficial by attenuating the mesenteric vascular hyporeactivity, by increasing the mesenteric blood flow decrease and by attenuating the injury in liver and spleen (Iskit et al., 1999; Kavuklu et al., 2000).

Thus, in our pursuit of a useful therapeutic intervention against sepsis, we attempted to investigate the effects of endothelin receptor blockade on the survival and organ injury in caecal ligation and puncture-induced sepsis model in mice, with particular emphasis on the effects of fluid resuscitation and the timing of the administration of bosentan.

2. Methods

2.1. Animals

Swiss albino mice (25–35 g) were obtained from the in-bred colony of the Laboratory Animal Husbandry Facility of the Department of Pharmacology, Hacettepe University Faculty of Medicine. The animals were housed under environmentally controlled conditions at 21±2 °C and 30–70% relative humidity with 12-h dark/12-h light illumination sequence (the lights were on between 0700 and 1900 h) with ad libitum access to tap water (drinking bottle) and standard pellet dairy chow (Korkutelim Yem Sanayii, Antalya, Turkey). The Guiding Principles in the Care and Use of Laboratory Animals together with The Recommendations from the Declaration of Helsinki were strictly adhered to during the execution of all the procedures described within this manuscript. This project was approved by the institutional Experimental Animal Care and Use Ethics Committee of Hacettepe University (Approval Number: 2004/3-7) before the commencement of any intervention.

2.2. Mouse model of polymicrobial sepsis

Polymicrobial sepsis was induced in mice by caecal ligation and puncture according to the detailed guiding of previous publications (Baker et al., 1983; Yang et al., 2002). In brief, mice were fasted overnight, but were allowed ad libitum access to drinking water prior to the experiment. The animals were then anaesthetized with chloralhydrate (400 mg kg^{-1}, i.p.) and a 1-cm midline incision was made. The caecum was then exposed, ligated just distal to the ileocaecal valve to avoid any intestinal obstruction, and punctured twice with a 22-gauge needle. The punctured caecum was gently squeezed to expel a small amount of fecal material and the abdominal incision was then closed in two layers by using atraumatic 4-0 silk sutures. Sham-operated animals underwent the same surgical procedure except that the caecum was neither ligated nor punctured. Some of the animals received normal saline (1 ml) subcutaneously immediately after the surgery which is essential for producing the hyperdynamic phase of sepsis during earlier stages in this experimental model (Yang et al., 2002).

2.3. Experimental protocols

This particular dose of bosentan (i.e., 30 mg kg^{-1}, i.p, per day) was chosen on the basis of our previous experience (Iskit et al., 1999; Iskit and Guc, 2001). In fact, we have previously utilized bosentan at three different doses of 3, 10 and 30 mg kg^{-1}, i.p. in other experimental models of sepsis, and we were quite familiar with its effects. Since the main emphasis of this study is on the timing of its administration after the surgery and the effects of fluid resuscitation in caecal ligation and puncture model, we have opted for the undisputably effective dose (i.e., 30 mg kg^{-1}, i.p., per day) of bosentan to be on the safe side. It is noteworthy to emphasize that the animals have received up to six doses of bosentan (30 mg kg^{-1}, i.p., per day) or its solvent if they have survived until the end of the observation period of 6 days (i.e., 144 h). Also by the help of this protocol, we were able to keep the number of animals that suffer at a possible minimum without losing the power of our statistical analyses which successfully passed through the ethical approval scrutiny.

At 5 or 20 h after caecal ligation and puncture, animals were treated with endothelin receptor antagonist bosentan or its solvent nonpyrogenic sterile saline (0.9% NaCl, w/v) at corresponding time points.

All drugs were prepared daily, dissolved in non-pyrogenic sterile saline and warmed to body temperature

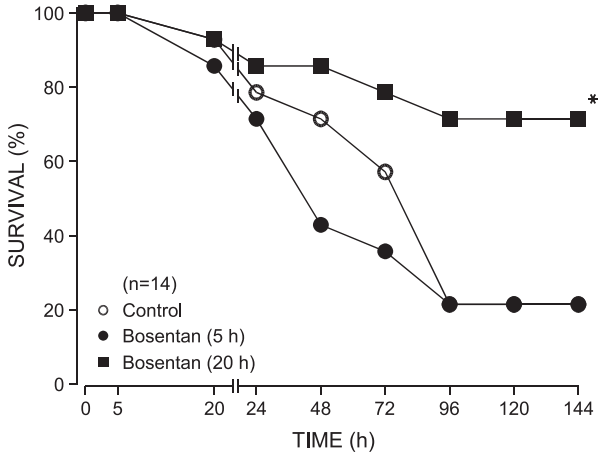

Fig. 1. Survival rates obtained from mice challenged with caecal ligation and puncture and resuscitated with 1 ml of saline immediately after the surgery. The animals were also given or bosentan (30 mg kg^{-1}, i.p.) or its solvent (saline 1 ml kg^{-1}, i.p.), 5 h or 20 h after the caecal ligation and puncture. "*n*" indicates the number of animals in each group. The asterisk (*) indicates significant difference ($P<0.05$, two-tailed value, Fisher's exact test) from the corresponding value obtained from control animals at 144 h.

(approximately 37 °C) before the injection. Drug solutions were kept in dark containers until injection in order to protect them from light-induced decomposition.

Survival rates were recorded until 144 h after caecal ligation and puncture but no additional deaths have occured in either of the groups after 96 h. The surviving animals were put down humanely at the end of the observation period and their organs were isolated.

2.4. Organ injury

The alterations in liver, spleen and kidney morphology (integrity) were examined by isolating the organs at 144 h after caecal ligation and puncture. The tissues were harvested accordingly, fixed in 10% neutral buffered formaldehyde solution and later embedded in paraffin. The tissue samples were then sectioned and stained with hematoxylin and eosin. The blinded pathologist evaluated the slides under conventional light microscopy and documented the majority of them by photographs.

2.5. Drugs used

Bosentan (Ro 47-0203) was kindly provided by Dr. Martine Clozel from Actelion, (Allschwill, Switzerland). Chloralhydrate, sodium chloride, hematoxylin (Merck, USA), eosin (Sigma, USA), formaldehyde (Carlo Erba, Italy), paraffin (Shandon, UK).

2.6. Statistical analysis

Fisher's exact test for 2×2 tables was used to compare the percent of survivals at selected (i.e., 144 h after caecal ligation and puncture CLP) time points. The differences were considered to be statistically significant when the two-tailed *P* value was less than 0.05.

3. Results

3.1. The effect of endothelin inhibition on survival in saline-resuscitated animals

In control animals, the overall survival was 21.4% at 144 h after caecal ligation and puncture (Fig. 1). When the animals were given bosentan 5 h after caecal ligation and puncture, the survival appeared to worsen at 24-, 48- and 72-h observation points but none of these values were significantly different than that of controls. Moreover, the overall survival has reached to the same value (i.e., 21.4%) at 96 h and remained so until 144 h. However, when bosentan was given 20 h after caecal ligation and puncture, the overall survival at 144 h was 71.4% which was significantly ($P=0.0213$) better than controls (or early bosentan treatment), implying the deleterious effects of endothelin peptides at relatively later stages of sepsis (Fig. 1).

3.2. The effect of endothelin inhibition on survival in nonresuscitated animals

In nonresuscitated control animals, the overall survival was 14.3% at 144 h after caecal ligation and puncture (Fig. 2). In contrast to resuscitated animals, the administration of bosentan has significantly improved the survival both at 5 h (78.6%, $P=0.0018$) and at 20 h (64.3%, $P=0.0183$) after caecal ligation and puncture, implying the deleterious

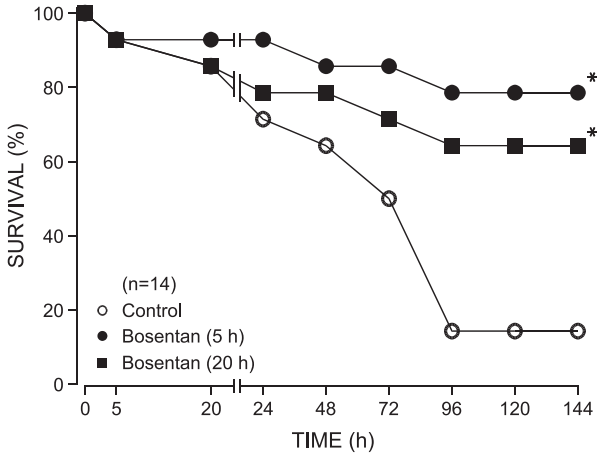

Fig. 2. Survival rates obtained from mice challenged with caecal ligation and puncture but not resuscitated with any fluid. The animals were also given or bosentan (30 mg kg^{-1}, i.p.) or its solvent (saline 1 ml kg^{-1}, i.p.), 5 h or 20 h after the caecal ligation and puncture. "*n*" indicates the number of animals in each group. The asterisk (*) indicates significant difference ($P<0.05$, two-tailed value, Fisher's exact test) from the corresponding value obtained from control animals at 144 h.

effects of endothelin peptides during the hypodynamic stages of sepsis (Fig. 2).

3.3. Histopathological examination of liver, spleen and kidney

From the morphological point of view, caecal ligation and puncture-induced septic shock produced severe haemocongestion in the liver parenchyma in addition to the inflammatory lymphocytic infiltration around the bile canaliculi together with the formation of minimal parenchymal injury in the form of spotty necrosis in liver and profound hydropic degeneration (Fig. 3A). In all spleen and kidney sections, there was significant congestion (Fig. 3B and C). When the animals were treated with endothelin receptor antagonist bosentan, liver architecture was well preserved (Fig. 3D) and the spleen (Fig. 3E) and kidney (Fig. 3F) were completely prevented from the histopathological injuries inflicted by caecal ligation and puncture.

4. Discussion

The main finding of this study was that the inhibition of endothelin receptors during the later (i.e., hypodynamic) phase of septic shock attenuated caecal ligation and puncture-induced mortality and attenuated the injury in the liver, spleen and kidney of mice which may suggest a novel therapeutic approach for human septic shock.

Current therapeutic modalities for septic shock failed to make a substantial impact on the high mortality. Septic shock remains the leading cause of death in noncoronary intensive care units, with a mortality rate ranging between 30% and 90% (Rangel-Frausto et al., 1995). Most of the therapeutical interventions are all uniformly based on the principal aim of combating the refractory hypotension by using volume replacement, antimicrobial chemotherapy, mechanical ventilation, oxygen therapy, large doses of vasoconstrictors and glucocorticoids, which do not offer consistent success (Iskit and Guc, 2003, 2004). Therefore,

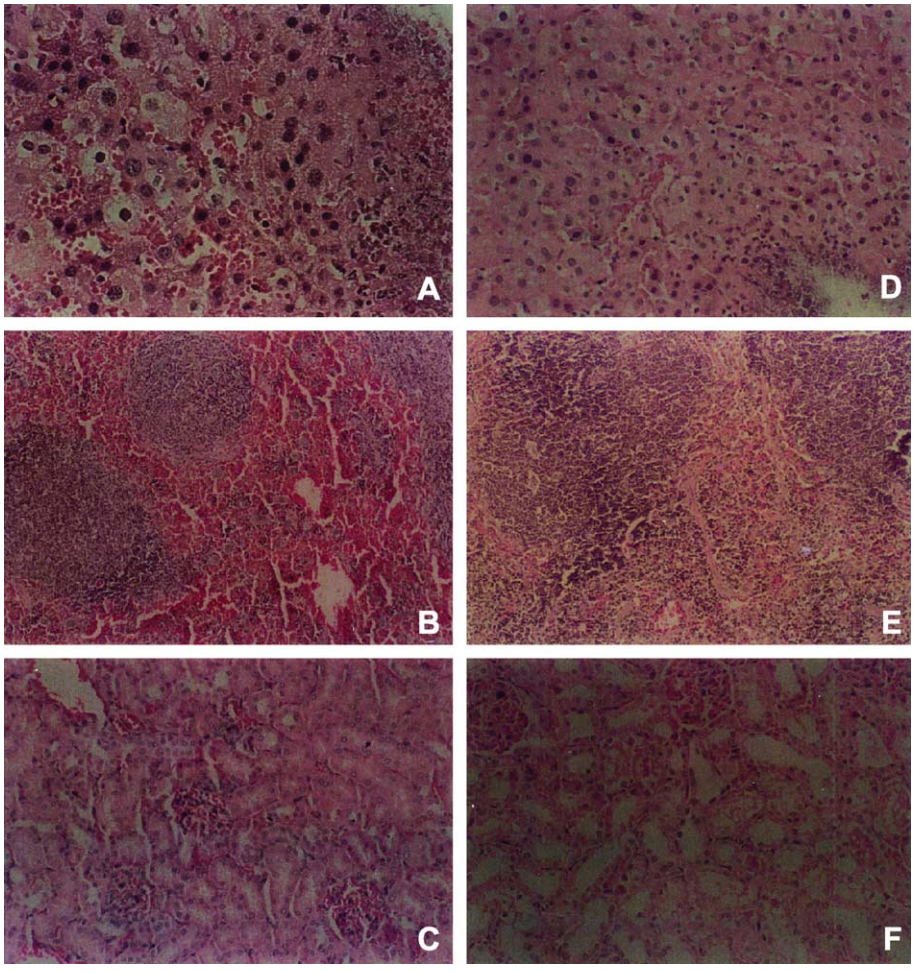

Fig. 3. The photographs on the left column depict histological sections of (A) liver, (B) spleen and (C) kidney obtained from mice challenged with caecal ligation and puncture and resuscitated with 1 ml of saline immediately after the surgery. On the right-hand column, the histopathological effects of bosentan (30 mg kg^{-1}, per day for 6 days, i.p.) treatment on the (D) liver, (E) spleen and (F) kidney of animals are given. (Hematoxylin and eosin staining, conventional light microscopy, magnification 20× or 40×).

lessons learned from previous studies and failure of the current approaches should stimulate researchers to find new treatment modalities. A very large number of biologically active substances are released into the circulation under conditions of endotoxaemia and sepsis. Nitric oxide and cytokines, such as tumour necrosis factor alpha, interleukin-1 are well established to be released during sepsis. Similarly, endothelin is gaining popularity as being regarded as one of the key mediator in systemic inflammatory response syndrome that lead to fatal multiple organ dysfunction (Wanecek et al., 2000; Iskit and Guc, 2001).

In a variety of pathological states, notably ischaemia-reperfusion injury, organ infarction and various shock states, there are substantial increases in the normally low circulating concentrations of endothelin. However, the serum levels of endothelin do not correlate with the severity of the pathological state as the secretion of endothelin peptides occurs in a polar fashion, with approximately 80% towards the abluminal side of the vessel (Wagner et al., 1992). In particular, endothelin release is stimulated by endotoxin (Sugiura et al., 1989) and endothelin peptides are increased in the circulation of sepsis patients (Weitzberg et al., 1991; Brauner et al., 2000). Studies have suggested that the release of endogenous endothelin during endotoxemia may help counteract severe hypotension, especially in the setting of nitric oxide synthase inhibition (Iskit and Guc, 2003, 2004). However, the increased levels of endothelin may lead to excessive vasoconstriction in peripheral vascular beds, and endothelin contributes to the dysfunction of multiple organs during endotoxemia, including liver, kidney and lung (Wanecek et al., 2000).

Investigators studied functional roles of endothelins in septic shock by using dual endothelin ET_A/ET_B receptor antagonists such as bosentan or SB209670 [(+)-1*S*,2*R*,*S*-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-prop-1-yloxyindane-2-carboxylate)], which provided conflicting results. Bosentan injection in septic pigs improved survival, cardiac index, restored the arterial pressure, enhanced systemic oxygen delivery, and acid–base status and completely abolished lipopolysaccharide-induced pulmonary hypertension and alterations in lung mechanics (Wanecek et al., 2000; Albertini et al., 2001). In contrast, another dual endothelin receptor antagonist SB 209670 is reported to aggravate the circulatory failure (Gardiner et al., 1995) as well as the renal and liver dysfunction caused by endotoxin in the rat (Ruetten et al., 1996). The reasons behind these differences between the results are not clear. Species differences, variations in haemodynamic profiles of various septic shock models, the in vivo effectiveness and selectivity of endothelin-receptor antagonists and the timing of the administration of these antagonists during the course of septic shock can be addressed. Despite the presence of considerable amount of data obtained from studies performed by endotoxin injection to experimental animals, little research has been conducted to demonstrate the effects of endothelin peptides in a clinically more relevant animal model of septic shock like caecal ligation and puncture.

In the present study, we demonstrated that that the timing of endothelin inhibition may significantly affect survival. Although septic shock is a highly complex pathophysiological state, the course of septic shock has different phases with different characteristics which need different (special) treatment strategies. In the mice model of polymicrobial sepsis as induced by caecal ligation and puncture, the cardiovascular response includes an early, hyperdynamic phase (if resuscitated) characterized by increased cardiac output, increased tissue perfusion, increased oxygen delivery and oxygen consumption, and decreased peripheral resistance. This is followed by a late, hypodynamic phase characterized by reduced microvascular blood flow in various tissues and decreased oxygen delivery, and increased peripheral resistance (Yang et al., 2002). In this context, the fluid resuscitation immediately after the surgery has helped mimic the clinical situation in which the septic patients are routinely given intravenous fluids. This point is further supported by previous studies which reported that the injection of 1 ml of normal saline immediately after caecal ligation and puncture in the rat is essential for producing the hyperdynamic response during the early stage of sepsis (Wang et al., 1991; Yang et al., 2002). However, in late sepsis, the inadequate tissue perfusion cannot meet both systemic and regional metabolic and oxygen demands. Because microcirculatory injury is considered as the fundamental mechanism for the development of organ dysfunction and multiple organ failure during sepsis (Parker et al., 1987), the primary objective in the management of sepsis is to maintain haemodynamic and cardiovascular stability and adequate tissue perfusion, and to meet the increasing metabolic and oxygen demands. The outcomes of this study seem to indicate that endothelin antagonists would be therapeutically beneficial in human endotoxic shock in this sense.

Another important problem in septic shock is the maldistribution of blood flow and insufficent oxygen supply to the tissues and organs (i.e., hypoperfusion). Liver, spleen and kidneys are the major target organs for sepsis-induced injury. Thus, increasing the blood flow to these organs must be the major aim of the treatment. The liver is, however, a highly important organ under septic conditions. The Kupffer cells constitute the largest population of tissue macrophages in the body and are of great immunological importance as they participate in the clearance of bacteria and toxins derived from a leaky septic gut. Endothelin receptor antagonism, by improving liver perfusion and thereby attenuating liver injury, may become an important tool in the treatment of septic shock in the future. According to the results of this study and other studies, dual endothelin receptor antagonist bosentan may be a good candidate for this purpose (Ruetten et al., 1996; Iskit et al., 1999). The beneficial effects of bosentan on kidney and spleen injury is

also important as these organs are the primarily affected crucial targets of the body in sepsis.

Therefore, we report here for the first time that antagonism of endothelin receptors in mice decreases caecal ligation and puncture-induced mortality and attenuates liver, spleen and kidney injury. On the basis of these results, we also suggest that the blockade of endothelin receptors during the late and/or hypodynamic phase of septic shock appears to be a promising novel strategy for the treatment of septic shock.

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