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The timing of endothelin and nitric oxide inhibition affects survival in a mice model of septic shock

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Abstract

The effect of endothelin and nitric oxide (NO) inhibition on survival from septic shock was investigated in male Swiss albino mice (20–40 g), with particular emphasis on the timing of the administration of their blockers after *Escherichia coli* endotoxin (lipopolysaccharide, O55:B5, 60 mg kg^{-1}, i.p.) challenge. Mice were injected with the endothelin receptor antagonist bosentan (30 mg kg^{-1}, i.p., either 2 or 12 h after endotoxin) alone or in addition to the NO synthase blockers L-canavanine (100 mg kg^{-1}, i.p.), N^G-nitro-L-arginine methyl ester (L-NAME, 3 mg kg^{-1}, i.p.) or aminoguanidine (15 mg kg^{-1}, i.p.), which were also given twice at 2 and 6 h after endotoxin. Control animals received saline, and survival rates in each group ($n = 10$) were recorded over 24 h at 6-h intervals. At 24 h, the survival rate was 10% in controls, but 30% (n.s.) and 70% ($P < 0.05$) in animals that received only bosentan at 2 and 12 h, respectively, indicating a relatively late involvement of endothelin in comparison to NO. In contrast, these figures were 70% ($P < 0.05$) and 80% ($P < 0.05$) at 12 h for L-NAME and L-canavanine, respectively, and 10% (n.s.) at 24 h, implying a relatively early involvement of NO compared to endothelin. Interestingly, survival in the aminoguanidine group (75% at 24 h, $P < 0.05$ vs. controls) was markedly higher than that in the L-NAME and L-canavanine groups, an effect that was attributed to mechanisms other than NO inhibition. Survival was better (60%, $P < 0.05$ vs. endotoxin alone) when bosentan was given at 2 h in combination with L-NAME, but the best outcome (90% survival, $P < 0.05$) was observed in animals when bosentan was given at 12 h and L-NAME was injected twice at 2 and 6 h. However, the statistical analysis revealed no significant additional beneficial effect of L-NAME coadministered with bosentan. Therefore, we conclude that NO is involved during the earlier phases of septic shock in comparison to a relatively late involvement of endothelin peptides, and that bosentan alone appears to be beneficial when administered at least 12 h after the endotoxin challenge in our mice model of septic shock. © 2001 Published by Elsevier Science B.V.

Keywords: Septic shock treatment; Endothelin; Nitric oxide (NO); Survival rate

1. Introduction

Septic shock is a serious circulatory disorder with an unacceptably high mortality rate of 30–90% (Rangel-Frausto et al., 1995), indicating an apparent inefficiency of its current treatment. Although it is widely accepted that the development of septic shock occurs in different phases with different characteristics (Groeneveld and Thijs, 1986), most of the therapeutic interventions are uniformly based on the principal aim of combating the refractory hypotension by using aggressive fluid infusions, large doses of vasoconstrictors and glucocorticoids (Baumgartner and Calandra, 1999), but these interventions do not offer consistent success (Parratt, 1997).

Despite some opposing views (Macmicking et al., 1995; Lefer, 1998), pivotal roles are attributed to endothelium-derived substances (Wort and Evans, 1999) such as endothelin (Pittet et al., 1991) and nitric oxide (NO) (Kilbourn, 1998) in sepsis-related syndromes, mainly on the basis of the outcome of animal experiments (Thiemermann, 1997) and on a limited number of clinical observations involving human subjects (Schilling et al., 1993).

In particular, endothelin release is stimulated by endotoxin (Sugiura et al., 1989), and the levels of endothelin peptides are increased in the circulation of patients with sepsis (Weitzberg et al., 1991), which may be a beneficial effect that may help maintain the blood pressure and organ perfusion during the initial phases of septic shock (Vemulapalli et al., 1991). However, excessive increases in the plasma levels of endothelin for longer periods evoke profound vasoconstriction in the splanchnic vascular bed, which is harmful (Ruetten and Thiemermann, 1996). Re-

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cently, bosentan, a non-specific endothelin ET_A and endothelin ET_B receptor antagonist with no intrinsic agonist activity, became available (Clozel et al., 1994), and its use makes it possible to abolish the deleterious effects of endotoxin such as mesenteric vasoconstriction together with spleen and liver injury (Iskit et al., 1999).

The case for the role of NO in endotoxaemia is similar. NO, produced in copious amounts by inducible NO synthase (Schulz et al., 1992), contributes significantly to the deleterious effects of endotoxin such as hypotension (Thiemermann and Vane, 1990), vascular hyporesponsiveness (Guc, 1992) to vasoconstrictors (Guc et al., 1990; Julou Schaeffer et al., 1990) and vasodilators (Guc et al., 1991). When the inducible enzyme is blocked selectively by using L-canavanine (Iyengar et al., 1987), mice challenged with a lethal dose of endotoxin show significant improvements in haemodynamic and metabolic parameters, as well as increased survival (Liaudet et al., 1996). However, non-selective NO synthase inhibition with N^G-substituted L-arginine analogues (e.g. N^G-nitro-L-arginine methyl ester: L-NAME) exacerbates endotoxin-induced organ ischaemia and mortality due to concomitant inhibition of the constitutive NO synthase (Hutcheson et al., 1990). Recent studies have shown that transgenic mice that overexpress the endothelial isoform of NO synthase are resistant to the deleterious effects of endotoxin (Yamashita et al., 2000) and that the endothelial isoform acts as the inducible isoform in producing venodilation in human beings during endotoxaemia (Bhagat et al., 1999). These findings highlight the potential hazards of focusing on increased NO production as the solitary target when addressing the underlying mechanisms of the rather high mortality rate of septic shock.

Therefore, the aim of this study was to investigate the effects of antagonist-induced inhibition of endothelin and NO on the survival of mice challenged with a lethal dose of endotoxin either alone or in combination. Because septic shock is composed of apparently distinct phases with diverse features, we injected the antagonists at different time points after endotoxin administration in an attempt to elaborate the significance of the timing of such interventions. A preliminary account of some data contained in this manuscript was presented in abstract form to the 13th Annual Meeting of The Surgical Infection Society of Europe, which was held on 25–27 May 2000, Nijmegen, The Netherlands (Iskit and Guc, 2000).

2. Methods

2.1. Animals

Eighty male Swiss albino mice (25–35 g) were obtained from the Laboratory Animal Husbandry Facility of the Department of Pharmacology, Hacettepe University Faculty of Medicine, and were housed under environmentally controlled conditions at 21 ± 2°C and 30–70% relative humidity with a 12-h dark/12-h light illumination sequence (the lights were on between 0700 and 1900 h) with access ad libitum to tap water (drinking bottle) and standard pellet dairy chow (Murat Yem Sanayi, Ankara, 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 implementation of all the procedures described in this manuscript.

2.2. General procedures

Endotoxin derived from *Escherichia coli* (lipopolysaccharide, O55:B5; 60 mg kg^{-1}, i.p.) or an equivalent volume (0.2 ml, i.p.) of non-pyrogenic sterile saline (NaCl 0.9%, w/v, dissolved in pyrogen-free distilled water) was given by intraperitoneal injection to animals without any additional resuscitative fluid administration. After the injection of endotoxin, animals were placed in separate cages and they were allowed to recover from the intervention under the standard conditions of the Laboratory Animal Husbandry Facility of the Department of Pharmacology, Hacettepe University, Faculty of Medicine, with minimal disruption.

2.3. Experimental protocols

This particular dose of endotoxin was chosen on the basis of our previous experience that revealed a mortality rate of at least 90% at 24 h in mice (Iskit et al., 1999). The doses and administration protocols for aminoguanidine (15 mg kg^{-1}, i.p., twice at 2 and 6 h after endotoxin; Laszlo et al., 1995), L-NAME (3 mg kg^{-1}, i.p., twice at 2 and 6 h after endotoxin; Wu et al., 1995), L-canavanine (100 mg kg^{-1}, i.p., twice at 2 and 6 h after endotoxin; Liaudet et al., 1996) and bosentan (30 mg kg^{-1}, i.p., at 2 h or 12 after endotoxin; Clozel et al., 1994) were chosen according to the information available in the literature. Control animals received the solvent (i.e. non-pyrogenic sterile saline) of the drugs used at corresponding time points.

All drugs were prepared daily, dissolved in non-pyrogenic sterile saline and warmed to body temperature (approximately 37°C) before injection. Drug solutions were kept in dark containers until injection in order to protect them from light-induced decomposition. Survival rates were recorded at 6-h intervals until 24 h after endotoxin challenge. In fact, the animals were followed at 24-h intervals for a further 120 h but no additional deaths occurred. Thus, the data used in this manuscript relate to the outcome of the first 24 h of observation in order to be on the safe side with regard to the clearance of drugs.

2.4. Drugs used

Endotoxin (lipopolysaccharide, *E. coli* O55:B5), aminoguanidine hemisulphate, L-NAME, and L-canavanine

hydrochloride were all purchased from Sigma (USA). Bosentan (Ro 47-0203) was kindly provided by Dr. Martine Clozel from Actelion, Allschwill, Switzerland.

2.5. Statistical analysis

Mantel and Haenszel Chi-square, a test that combines a series of 2×2 tables formed at different points in a survival distribution, was used to test the overall significance between the curves (Dawson-Saunders and Trapp, 1989). In the presence of a significant difference between the curves for different treatment groups, further analysis was performed by using Fisher's exact test for 2×2 tables to compare the percent survival at selected (i.e. 24 h after endotoxin administration) time points. The differences were considered to be statistically significant when the two-tailed P value was less than 0.05 and the conclusions were then drawn accordingly.

3. Results

3.1. The effect of endothelin inhibition alone on survival

None of the control animals died during the first 6 h after endotoxin administration but the overall survival was 10% at 24 h (Fig. 1). When the animals were given bosentan 2 h after the administration of endotoxin, 30% of them died within the first 6 h, which was not significantly different than controls. Similarly, the overall survival at 24 h (i.e. 30%) was not significantly different from that of the controls either. However, when bosentan was given 12 h after endotoxin, the overall survival at 24 h was 70%, which was significantly ($P = 0.01977$) better than that of the controls, implying the involvement of endothelin peptides at a relatively late stage of septic shock (Fig. 1).

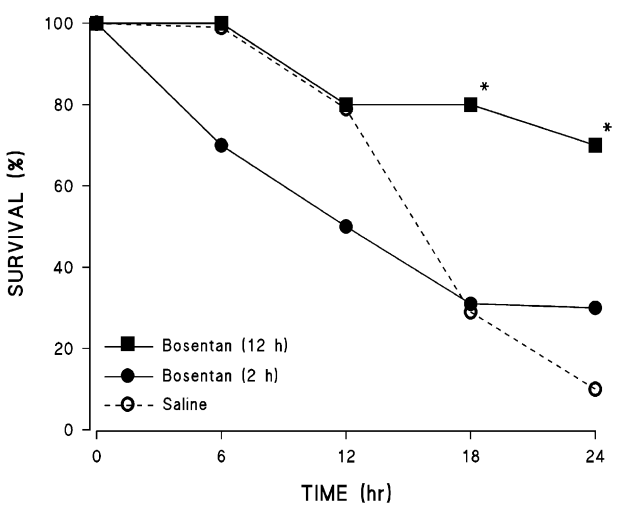

Fig. 1. Survival rates for mice challenged with 60 mg kg^{-1} endotoxin intraperitoneally and treated with either the solvent (saline 1 ml kg^{-1}, i.p.) or bosentan (30 mg kg^{-1}, i.p., 2 or 12 h after endotoxin). $n = 10$ for each group. * indicates significant difference ($P < 0.05$, two-tailed value, Fisher's exact test) from the control values at corresponding time points.

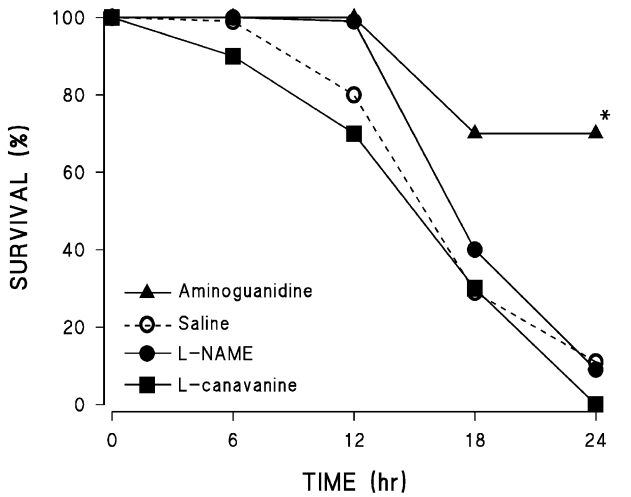

Fig. 2. Survival rates for mice challenged with 60 mg kg^{-1} endotoxin intraperitoneally and treated with either the solvent (saline 1 ml kg^{-1}, i.p.) or aminoguanidine (15 mg kg^{-1}, i.p., at 2 and 6 h after endotoxin), L-canavanine (100 mg kg^{-1}, i.p., at 2 and 6 h after endotoxin) or L-NAME (3 mg kg^{-1}, i.p., at 2 and 6 h after endotoxin) as post hoc interventions. $n = 10$ for each group. * indicates significant difference ($P < 0.05$, two-tailed value, Fisher's exact test) from the control value at corresponding time point.

3.2. The effect of NO inhibition alone on survival

When the animals were given L-NAME (a non-specific inhibitor of NO synthase) or L-canavanine (a relatively specific inhibitor of the inducible form of NO synthase) twice at 2 and 6 h after endotoxin injection, the survival curves were closely parallel to that of controls: the overall survival rate at 24 h was not significantly different from that of the controls (Fig. 2). However, when the animals were treated with another inducible NO synthase inhibitor, aminoguanidine, twice at 2 and 6 h after endotoxin injection, the survival at 24 h was 70%, which was significantly ($P = 0.01977$) different from the corresponding control value (Fig. 2).

3.3. The effect of both endothelin and NO inhibition on survival

Fig. 3 shows the results of experiments in which L-NAME and bosentan were given in combination after the animals received endotoxin. When bosentan was given at 2 h after endotoxin injection in addition to L-NAME (administered twice at 2 and 6 h after endotoxin), the overall survival at 24 h was 60%, which was marginally better ($P = 0.05728$) than that of controls. However, the administration of bosentan at 12 h after endotoxin injection in addition to L-NAME (administered twice at 2 and 6 h after endotoxin) resulted in a survival rate of 100% ($P = 0.00310$

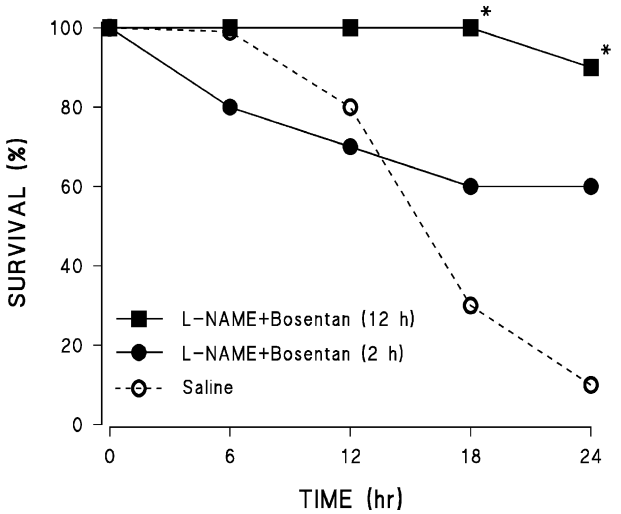

Fig. 3. Survival rates for mice challenged with 60 mg kg^{-1} endotoxin intraperitoneally and treated with either the solvent (saline 1 ml kg^{-1}, i.p.) or two different combinations of L-NAME (3 mg kg^{-1}, i.p., at 2 and 6 h after endotoxin) and bosentan (30 mg kg^{-1}, i.p., either at 2 or 12 h). $n = 10$ for each group. * indicates significant difference ($P < 0.05$, two-tailed value, Fisher's exact test) from the control values at corresponding time points.

vs. controls) at 18 h and 90% ($P = 0.00109$ vs. controls) at 24 h, which was much better than that of controls (Fig. 3).

Despite the fact that superior survival rates were obtained with the combined administration of bosentan and L-NAME in this study, a further comparative statistical analysis of the curves obtained from animals that received only bosentan at 12 h after endotoxin administration (Fig. 1, line with solid squares) vs. that obtained from animals that received L-NAME twice at 2 and 6 h after endotoxin in addition to bosentan (Fig. 3, line with solid squares) indicated no significant difference ($P = 0.4062$) in their overall survival. Moreover, the survival at 24 h after endotoxin administration was not significantly different either (i.e. bosentan alone: 70% vs. L-NAME + bosentan: 90%, $P = 0.58204$).

4. Discussion

These results indicate for the first time that, in a mice model of septic shock with a rather high mortality rate of 90% at 24 h, it is possible to improve survival by inhibiting endothelin and NO after endotoxin challenge. This survival rate can be sustained by inhibition of endothelin peptides with bosentan. Another feature of this study is that it reveals the unusual efficacy of a post hoc intervention on survival for the first time, in contrast to numerous earlier studies that reported rather inconsistent success with various prophylactic measures.

The cascade of events that lead to the highly fatal outcome of septic shock is believed to be triggered by the entrance of bacterial wall components, mainly the Gram (−) endotoxin, into the systemic circulation (Glauser et al., 1991), although other bioactive components, such as exotoxin, lipoteichoic acid and peptidoglycan complexes (Bone, 1991) or bacterial DNA (Sparwasser et al., 1997), can also produce a similar outcome. Independent of the type of initiating events, most of the patients that eventually die from septic shock exhibit similar symptoms that can be categorized into at least four distinct clinical phases, namely, preshock, hyperdynamic, hypodynamic and irreversible phases (Groenveld and Thijs, 1986). Among these, the hyperdynamic phase and the hypodynamic phase, display markedly opposite characteristics with regard to the cardiovascular system. During the hyperdynamic phase, which is also named as the "early", "warm" (Nishijima et al., 1973) or "compensated" (Abboud, 1988) phase, cardiac output is elevated and systemic vascular resistance is low (Hardaway, 1980), indicating the predominance of vasodilator mechanisms (Thijs and Groeneveld, 1987). In contrast, during the hypodynamic, "late", "cold" (Nishijima et al., 1973) or "decompensated" (Abboud, 1988) phase, cardiac output is low and systemic vascular resistance is increased (Groeneveld et al., 1987) due to circulating vasoconstrictors (Lee, 1974). There is also an increase in vascular permeability resulting from fluid flux into the extravascular space (Van Lambalgen et al., 1987).

When the characteristics of these two phases are overviewed in general terms, NO-related mechanisms appear to be one of the main players during the early phase whereas the pharmacological profile of the endothelin peptides is sufficient to explain the features of the subsequent phase. Therefore, our way of attacking the familiar problem of low survival in animal models of septic shock by inhibiting NO during the "early" stage combined with relatively "late" inhibition of endothelin in this study appears to be the correct approach. Although the timing of these interventions was selected arbitrarily, the fact that the activity of the inducible NO synthase reaches its maximum within 2–6 h after endotoxin challenge (Schulz et al., 1992; Gardiner et al., 1995) and the adequate plasma half-life of bosentan (Clozel et al., 1994) validate our present experimental protocol.

Our present results with post hoc inhibition of endothelin by using bosentan are in agreement with those of previous studies that reported some beneficial effects as a result of its ad hoc inhibition in pigs (Weitzberg et al., 1996) and in rats (Mitolo-Chieppa et al., 1996). Since endothelin is the most potent vasoconstrictor substance known to date (Yanagisawa et al., 1988) and ischaemia in various organs contributes significantly to the high mortality rate in sepsis-related syndromes (Takala, 1996), it is possible that the beneficial effects of endothelin inhibition reside in its role in triggering the self-perpetuating vicious cycle of events resulting from intense vasoconstriction in various organ systems (Guc, 1999). In other words, endothelin, released in patients with sepsis in response to endotoxin (Sugiura et al., 1989), may help maintain blood

pressure and organ perfusion, effects which are beneficial during the initial phases of septic shock, whereas an excessive increase in the plasma levels of endothelin for longer periods evokes profound vasoconstriction that may be responsible for organ injury (Iskit et al., 1999) and mortality. Indeed, a recent study suggested the use of elevated levels of endothelin-1 in the circulation as an early predictor of mortality in patients with septic shock (Brauner et al., 2000).

The lack of any consistent beneficial effect of NO inhibition alone with regard to survival in the current study suggests that mechanisms in addition to excessive NO formation are largely responsible for endotoxin-induced mortality. This conclusion is supported by earlier results showing that non-selective or selective NO synthase inhibitors either do not influence survival (Macmicking et al., 1995) or enhance mortality (Wu et al., 1995). It is also possible to explain the apparent ineffectiveness of L-NAME and L-canavanine in enhancing survival by their inadequate final concentrations at their sites of action. However, this possibility can be ruled out because L-NAME, administered according to the same protocol of the present study (i.e. 3 mg kg^{-1}, i.p., twice at 2 and 6 h after endotoxin), can inhibit both the constitutive and the inducible isoforms of NO synthase in biochemical bioassays performed in vitro with tissue homogenates obtained from endotoxin-treated animals (Wu et al., 1995). Similarly, the dose of L-canavanine was sufficient because the results of others (Liaudet et al., 1996) and our previous studies demonstrated that at half of the dose (i.e. 100 mg kg^{-1}, i.p.) used in the present study (i.e. 100 mg kg^{-1}, i.p., twice at 2 and 6 h after endotoxin), L-canavanine is capable of inhibiting the anti-arrhythmic effects of endotoxin 4 h after its administration (Iskit et al., 1997). Moreover, at one tenth (i.e. 20 mg kg^{-1}, i.p.) of the total dose used in the present study, L-canavanine could also cause significant injury to the spleen in mice (Iskit et al., 1999).

In contrast to the ineffectiveness of solitary usage of L-NAME or L-canavanine, aminoguanidine alone provided a significant protection against endotoxin-induced mortality. Although categorized as a highly selective inhibitor of both the expression and the activity of inducible NO synthase (Thiemermann, 1998), its beneficial effect on survival in the present study can be partially explained by its protective effect against endotoxin-induced mesenteric ischaemia (Kavuklu et al., 2000) rather than its NO-blocking property. In order to justify this deduction, we would like to emphasize that, contrary to the widely accepted "endotoxin-NO overproduction" dogma that provides a blanket explanation for all of the effects of endotoxin in various vascular beds, endotoxin induces mesenteric vasoconstriction and this can be blocked by the so-called selective inducible NO synthase blocker aminoguanidine (Kavuklu et al., 2000). Since it is impossible to explain endotoxin-induced mesenteric vasoconstriction simply by endotoxin-induced overproduction of the well-established vasorelaxing mediator NO, aminoguanidine must have exerted its beneficial effect by using mechanisms other than inducible NO synthase blockade, for example inhibition of histamine (Bieganski et al., 1983) and polyamine catabolism (Seiler et al., 1985) or blockade of catalase or iron-/copper-containing enzymes and the formation of advanced glycolysation end-products (Ou and Wolff, 1993), as also suggested in a previous study (Wu et al., 1995).

Therefore, although the quest for a panacea for septic shock is hampered by the constant clinical failure of various interventions proven to be beneficial in animal models (Thiemermann., 1997), a post hoc intervention turned out to be profoundly effective in improving survival. Although we are aware that (i) the present data were also obtained from an experimental animal model of septic shock, (ii) tumour necrosis factor alpha is released with interleukin-1 (Glauser et al., 1991) within minutes after endotoxin exposure (thus, they are indeed the "early" mediators of sepsis) and (iii) high mobility group-1 protein that acts 8–32 h after endotoxin challenge deserves to be tagged as a "late mediator" (Wang et al., 1999), our present results showing the beneficial effect of inhibition of NO production during the early phases of sepsis in combination with blockade of endothelin receptors at a later stage suggest that this may be a promising approach to the treatment of septic shock.

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