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Terbutaline Prevents Vascular Hyporesponsiveness to Norepinephrine in Peritonitis-induced Septic Rats

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Background: Impaired vascular responsiveness to vasoconstrictors is often seen in sepsis, in particular in septic shock. Nitric oxide and superoxide anion (O_2^-) usually play an important role in mediating vascular hypo-responsiveness to vasoconstrictors in sepsis. The aim of this study was to determine whether terbutaline, a known cyclic-AMP agonist, can restore vascular hypo-responsiveness in cecal ligation and puncture (CLP)-induced sepsis models. **Methods:** Male adult Wistar rats received CLP or sham operations followed by the intravenous administration of saline or terbutaline (0.3 mg/kg at 3 and 9 h after CLP). At 0, 9 and 18 h after CLP, changes of arterial blood pressure, vascular responsiveness to norepinephrine (NE) and plasma nitrite/nitrate levels were examined. At 18 h after CLP, the animals were sacrificed and their thoracic aortae were immediately exercised to analyze O_2^- levels. **Results:** Vascular responsiveness to NE was impaired in CLP-treated rats. The administration of terbutaline did not alter vascular responsiveness to NE in sham-operated rats but significantly mitigated the vascular hypo-responsiveness to NE in CLP-treated rats. In addition, increased plasma nitrite/nitrate levels caused by CLP were suppressed by terbutaline. However, this had no significant effect on aortic O_2^- levels in CLP-treated rats. **Conclusion:** This study demonstrates that CLP-induced vascular hyporesponsiveness to NE is partially restored by treatment of rats with terbutaline, suggesting that terbutaline could be a new therapeutic agent to treat patients with septic shock resulting from impaired vascular responsiveness to vasoconstrictors.

Key words: β-adrenergic agonist, nitric oxide, superoxide anion, vascular hyporesponsiveness, sepsis

INTRODUCTION

Sepsis causes progressive systemic hypotension which is resistant to vasoconstrictors and leads to abnormal tissue perfusion and then organ failure. Despite recent advances in intensive care, the mortality rate in septic shock with multiple organ failure still exceeds 50%.[1] If adequate fluid volume resuscitation is insufficient in patients with septic shock, vasoactive and/or inotropic agents are always required to maintain arterial blood pressure, cardiac output and tissue vascular perfusion.[2] However, so far, the improvement of severe hypotension and hypoperfusion in sepsis is limited by hyporesponsiveness to endogenous and exogenous catecholamines such as norepinephrine (NE).

The mechanisms underlying this adrenoceptor hyporesponsiveness in sepsis are unclear, but overt production of nitric oxide (NO), principally by the calcium-independent inducible isoform of NO synthase (iNOS), has been implicated in animal experiments.[3] This is based on the fact that selective inhibitors of iNOS partly reverse the vascular hyporesponsiveness in a number of *in vivo* and *in vitro* animal models of endotoxic shock.[4,5] Concomitant with enhanced NO production, bacterial endotoxin or inflammatory cytokines also increase cellular superoxide anion (O_2^-) production, which can inactivate biologic mediators, including catecholamines.[6] The endotoxin-induced hyporesponsiveness to catecholamines can also be restored by high doses of the anti-oxidant vitamin C,[7] suggesting that reactive oxygen species also contribute to the pathogenesis of adrenoceptor hyporesponsiveness in sepsis.

Terbutaline, a selective β_2-adrenoceptors agonist, has been clinically used for the long-term treatment of obstructive airway diseases and for the treatment of acute bronchospasm.[8] Terbutaline binds to membrane-bound

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beta-receptors and exerts its effect through a stimulatory G protein that activates the adenylate cyclase, resulting in the formation of cyclic adenosine monophosphate (cAMP).[9] cAMP subsequently activates protein kinase, which controls many biochemical events through the phosphorylation of target proteins.[10] Moreover, we recently demonstrated that terbutaline attenuates severe hypotension and vascular hyporesponsiveness to NE in animals with endotoxic shock.[11-13]

For further study in clinically relevant models of sepsis, our study was designed to examine whether vascular responsiveness to NE could be reversed by terbutaline in a cecal ligation and puncture (CLP) rat model. In addition, we further investigated whether the reduction of NO and O_2^- contributes to the restoration of terbutaline in adrenoceptor hyporesponsiveness. The current peritonitis model produced by CLP reproduces many of the clinical features of human septic shock.[14] Therefore, the CLP procedure allows us to study alteration in organ function and the underlying mechanisms during different hemodynamic stages of polymicrobial sepsis.

METHODS

All work was approved by the institutional and local Committee on the Care and Use of Animals (National Defense Medical Center, Taipei, Taiwan, R.O.C.). Male adult Wistar rats (10-12 weeks old, 280-350 g body weight) were used in this study.

Surgical procedures and experimental protocol

All animals were anesthetized by intraperitoneal injections of sodium pentobarbital (40-50 mg/kg). The left carotid artery and the right jugular vein were cannulated and exteriorized to the back of the neck for hemodynamic measurements and drug administration, respectively. The cannulated rats were allowed to recover to normal condition overnight. After baseline (i.e. time 0) hemodynamic measurements and blood withdrawal, the rats were then randomly assigned into two groups: septic and sham-operated (SOP) rats. The intraperitoneal sepsis was induced by a CLP as described by Wichterman et al.[14] Briefly, the rats were first anesthetized with sodium pentobarbital (30-40 mg/kg, i.v.), then a small midabdominal incision was performed and the cecum was exposed. The cecum was then isolated and ligated with a 3-0 silk ligature just distal to the ileocecal valve, punctured twice at opposite ends with an 18-gauge needle, and returned into the abdominal cavity. The SOP rats underwent the same surgical procedure except that the cecum was neither ligated nor punctured. Afterward, CLP and SOP rats were intravenously treated with saline or terbutaline (0.3 mg/kg at 3 and 9 h after surgery).

At baseline and 9 and 18 h after CLP, mean arterial pressure (MAP) and heart rate were measured by a pressure transducer (P23ID, Statham, Oxnard, CA, U.S.A.) and displayed on a Gould model TA5000 polygraph recorder (Gould Inc., Valley View, OH, U.S.A.). Following the recording of hemodynamic parameters, animals were given NE (1 μg/kg, i.v.) to examine their vascular reactivity. In order to normalize the baseline value of pressor responses to NE for all groups, we calculated the value of pressor responses to NE at the baseline of each group as 100%. Blood was drawn from the carotid artery at 0, 9, and 18 h after CLP to determine plasma levels of nitrite/nitrate. Each volume of blood removed was immediately replaced by the injection of an equal volume of sterile saline. The animals were sacrificed by pentobarbital overdose followed by the harvesting of thoracic aortae to analyze O_2^- levels 18 hours after the CLP or sham operation.

Determination of plasma nitrite/nitrate levels

It is noted that the NO concentration in plasma depicted in the study is actually the total nitrite/nitrate concentration in plasma. With this method, as previously described, nitrate is reduced to NO via nitrite.[15] Briefly, 30 μl of plasma stored at -20 °C and tissue homogenates stored at -80 °C were thawed and de-proteinized by incubation with 95% ethanol (4 °C) for 30 min. The samples were subsequently centrifuged at 16,000 g for 5 min. The supernatant fraction (6 μl) was added to a reducing agent (0.8% vanadium (III) chloride in 1 N HCl) in the purge vessel. Nitrates in the samples were reduced to NO, which was stripped by using helium pure gas. The NO was then drawn into the Nitric Oxide Analyzer (Sievers 280 NOA, Sievers Instruments, Boulder, CO, U.S.A.). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate (Sigma Chemical Co., St. Louis, MO, U.S.A.).

Measurement of aortic O_2^- production

The thoracic aorta was isolated and removed rapidly after euthanasia. The aorta was carefully trimmed of extravascular tissues and then cut into rings of 5-mm width. Then, the tissues were incubated with warmed (37 °C) Krebs–HEPES buffer which were aerated with 95% O_2/5% CO_2 for 30 min and then transferred to scintillation plates. These scintillation plates containing Krebs–Hepes buffer with 1.25 mM lucigenin (final volume of

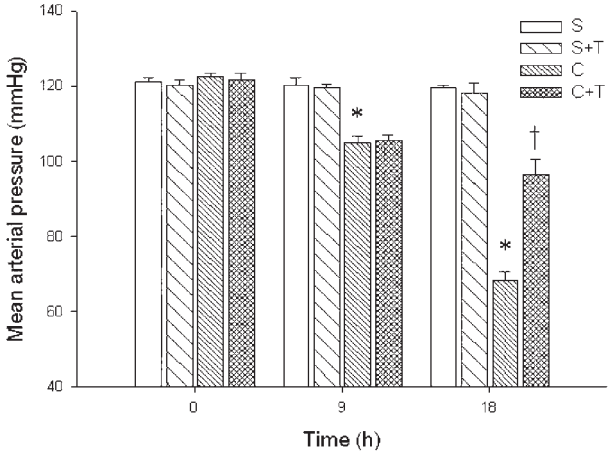

Fig. 1 Alterations in mean arterial pressure in sham-operated (SOP) animals treated with saline (S, n = 10) or terbutaline (S+T, n = 4), and septic animals treated with cecal ligation and puncture (CLP) plus saline (C, n = 10) or terbutaline (C+T, n = 10) during the experimental period. Data are expressed as mean±SEM. *P < 0.05, CLP vs. SOP rats; †*P* < 0.05, with versus without drugs in CLP rats.

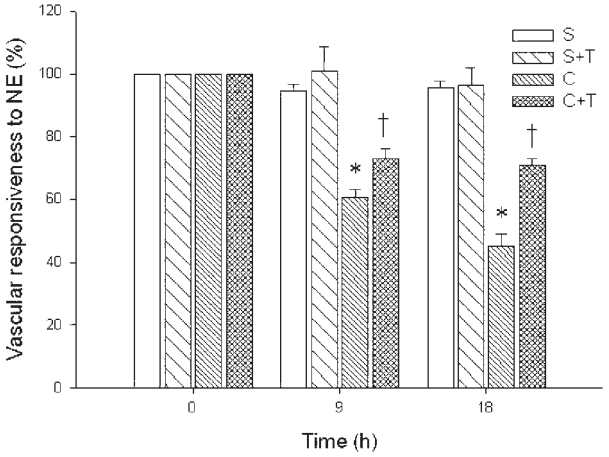

Fig. 2 Alterations in vascular responsiveness to norepinephrine (NE) in sham-operated (SOP) animals treated with saline (S, n = 10) or terbutaline (S+T, n = 4), and septic animals treated with cecal ligation and puncture (CLP) plus saline (C, n = 10) or terbutaline (C+T, n = 10) during the experimental period. Data are expressed as mean±SEM. *P < 0.05, CLP vs. SOP rats; †P < 0.05, with versus without drugs in CLP rats

250 μl) were placed into a microplate luminometer (Hidex Microplate Luminometer, Turku, Finland). Counts were obtained at 15-sec intervals at room temperature. All tissues were then dried in a 90 °C oven for 24 h. The results were expressed as counts/sec/mg (dry weight).

Statistical analysis

The data are presented as mean±SEM of *n* determinations, where n represents the number of animals studied. Statistical evaluation was performed by using analysis of variance (ANOVA) followed by a Bonferroni *post hoc* correction test. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

As shown in the results in Fig. 1, the baseline values for MAP were comparable in all groups. The SOP animals treated with saline exhibited stable MAP during the experimental period, but CLP led to significantly substantial, time-dependent attenuations in MAP (Fig. 1) during the experimental period. About half of the baseline MAP was decreased at 18 h following CLP. However, the administration of terbutaline significantly prevented hypotension at 18 h after CLP (Fig. 1). The SOP animals treated with terbutaline exhibited no significant changes in MAP during the experimental period, and there was no significant difference compared with the SOP group.

The baseline values for vascular response to NE were comparable in all groups. The SOP animals treated with saline exhibited stable vascular response to NE during the experimental period, but CLP led to significantly substantial, time-dependent attenuations in vascular response to NE from 9 h to 18 h after surgery (Fig. 2). The administration of CLP animals with terbutaline significantly attenuated vascular hyporesponsiveness to NE at 18 h after surgery (Fig. 2). The SOP animals treated with terbutaline exhibited no significant changes in vascular response to NE during the experimental period, and there was no significant difference compared with the SOP group.

The basal plasma level of nitrite/nitrate was not significantly different among the groups studied. No significant changes in plasma level of nitrite/nitrate were observed during the experimental period in SOP rats treated with saline. However, there was a significant elevation in plasma nitrite/nitrate level in the rats, reaching about 10 fold at 18 h after CLP (Fig. 3). The increase in plasma nitrite/nitrate levels at 18 h after CLP was significantly attenuated by terbutaline (Fig. 3). However, the SOP animals treated with terbutaline exhibited no significant changes in plasma nitrite/nitrate levels during the experimental

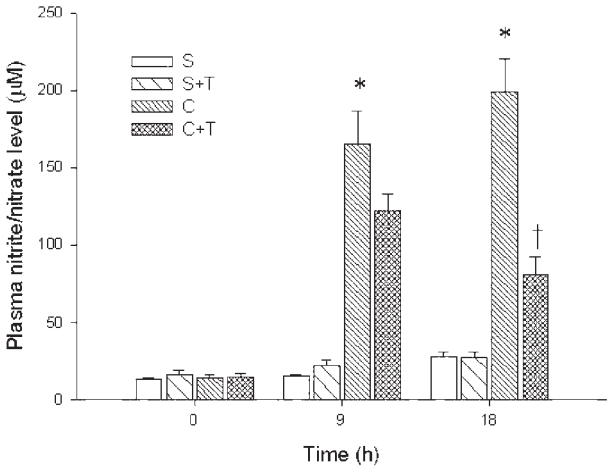

Fig. 3 Alterations in plasma nitrite/nitrate levels in sham-operated (SOP) animals treated with saline (S, n = 10) or terbutaline (S+T, n = 4), and septic animals treated with cecal ligation and puncture (CLP) plus saline (C, n = 10) or terbutaline (C+T, n = 10) during the experimental period. Data are expressed as mean±SEM. *P < 0.05, CLP vs. SOP rats; †P < 0.05, with versus without drugs in CLP rats.

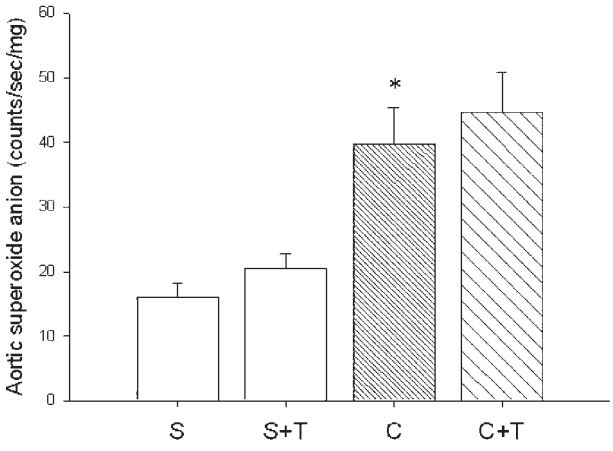

Fig. 4 Aortic superoxide level in sham-operated (SOP) animals treated with saline (S, n = 10) or terbutaline (S+T, n = 4), and septic animals treated with cecal ligation and puncture (CLP) plus saline (C, n = 10) or terbutaline (C+T, n = 10) at 18 h after surgery. Data are expressed as mean±SEM. *P < 0.05, CLP vs. SOP rats.

period, and there was no significant difference compared with the SOP group.

The basal production of O_2^- was detectable in the aortic ring (Fig. 4) obtained from the SOP rats. The administration of SOP rats with terbutaline had no significant effect on aortic O_2^- levels. In contrast, the O_2^- levels in aortic rings were significantly increased in rats at 18 h after CLP. However, the treatment of CLP rats with terbutaline did not inhibit the production of O_2^- in the aortic rings (Fig. 4).

DISCUSSION

In the present study, we demonstrated that peritonitis-induced sepsis was able to reproduce the impairment of vasoconstriction which often manifests in other in vitro and *in vivo* studies.[16-18] In addition, consistent with results shown in previous studies of endotoxic shock,[11-13] CLP-induced sepsis significantly decreased arterial blood pressure and impaired the vascular response to NE, and this hypotension and impairment of vascular function were prevented by the post-treatment of CLP rats with terbutaline. Bases on this study, we suggest that the beneficial effects of synthetic β_2-adrenoceptor agonist are attributed to the inhibition of increased nitrite/nitrate production in the plasma caused by CLP, but not to the attenuation of enhanced O_2^- level in the aortic vessel.

The development of CLP from peritonitis to sepsis presumably begins with polymicrobial bacteremia. The endotoxin, which is embedded in the outer walls of Gram-negative or -positive bacteria, activates the Toll-like receptors of mononuclear leukocytes and induces a signaling pathway involved in the activation of nuclear factor-κB (NF-κB).[19] Proinflammatory cytokines, transcriptionally activated by NF-κB, indirectly activate iNOS leading to excessive generation of NO.[20] NO and its derivative, peroxynitrite ($ONOO^-$, the product formed from the interaction between NO and O_2^-), primarily exert microbial killing during inflammation.[3] On the other hand, NO has the potential to induce vasodilatation due to its interaction with soluble guanylate cyclase, which increases the cyclic guanylyl 3',5'-monophosphate (cGMP) in the vascular smooth muscle cell.[21] Smooth muscle relaxation of NO is also known to mediate hyperpolarization of the smooth cell membrane by activating K^+ channels,[22] and decreasing the intracellular Ca^{2+} level.[23] Therefore, enhanced plasma levels of nitrite/nitrate (breakdown products of NO) in the present study may contribute to severe hypotension and vascular hyporesponsiveness to vasoconstrictors. Recently, it has been reported that exposure of NE to NO leads to 6-nitronorepinephrine, which has poor vasoactivities.[24] Concomitant with the enhanced production of NO, bacterial endotoxin

or proinflammatory cytokines may activate neutrophils to produce a number of reactive oxygen species, such as O_2^-, hydrogen peroxide and hydroxyl radicals, causing inappropriate organ damage.[25,26] In agreement with a previous experiment,[27] our data shows that CLP causes elevated levels of O_2^- in the aortic rings. In addition, O_2^- and $ONOO^-$ also autoxidize NE to an adrenochrome, which has poor vasoactivities.[6,28] The poor vasoactivity of these derivatives nitrated by NO or oxidized by O_2^- and $ONOO^-$ reveals the involvement of oxidants in the pathogenesis of septic shock.

Vascular β-adrenoceptors were originally classified as β_2, and this appears to be the predominant type in rat aorta.[29] β_2-adrenoceptor-mediated vasodilatation has been regarded as endothelium-independent in accordance with activation of adenylyl cyclase leading to a subsequent increase in intracellular cAMP concentration.[30] The stimulation of endothelial β_2-adrenoceptor may induce NO release, which then evokes relaxation via an increase in smooth muscle cGMP.[31] In addition, some potassium channels may be involved in mediating the β-adrenoceptor relaxant response via cAMP/cGMP-independent G-protein activation.[32,33] In the present study, we used the maximal dosage of terbutaline, and this did not affect any hemodynamic parameters in SOP rats during the experimental period but attenuated the severe hypotension and restored vascular hyporesponsiveness to NE caused by CLP. Therefore, the protective effects of terbutaline in septic shock may be related with the transcriptional regulation of proinflammatory mediators. This is based on the fact that β_2-adrenoceptor agonists inhibit the expression of intercellular adhesion molecule-1, CD40 and CD14 on monocytes, and this adrenoceptor agonist activity is inhibited by the selective β_2-adrenoceptor antagonist, butoxamine.[34]

It has been shown that LPS-induced stimulation of proinflammatory cytokines and iNOS probably involve NFκB cascades,[20] and cAMP may modulate this process when elevated following β_2-adrenoceptor stimulation.[35,36] In cultured cells, accumulating intracellular cAMP after inhibition of phosphodiesterase IV contributes to several anti-inflammatory parameters including suppression of microglial proliferation, O_2^- and NO production and the expression of TNF-α.[37] Our data suggests that terbutaline significantly attenuated the increased plasma nitrite/nitrate level caused by CLP, which may be associated with the restoration of CLP-induced vascular hyporesponsiveness to NE following terbutaline treatment. However, contrary to our previous study on endotoxic shock,[12] terbutaline did not reduce the O_2^- level in the aortic rings in this polymicrobial sepsis model.

In conclusion, the present study demonstrates the potential vasoprotective properties of terbutaline in peritonitis-induced polymicrobial sepsis. Given the important role of adrenoceptor hyporeactivity in the development of septic organ dysfunction, it is possible that preserved vascular responsiveness during sepsis by terbutaline may have contributed to the improvement of the outcome. This would be compatible with previous animal studies in which sepsis-related mortality was significantly reduced by terbutaline. However, whether this effect also exists in other β_2-adrenoceptor agonists or not remains to be elucidated.

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