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Effect of Hydroxocobalamin on Contractile Responses to Phenylephrine during Administration of Inhalational Anesthetics in Lipopolysaccharide-Treated Rat Aortae*

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ABSTRACT

The hemodynamic changes in septic patients produced by inhalational anesthetics are sufficient to threaten the anesthesiologists. The effect of hydroxocobalamin, a vitamin B_{12a}, on contractile responses to phenylephrine during administration of inhalational anesthetics were evaluated in aortic ring preparations obtained from LPS-treated rats.

The sepsis was developed by intraperitoneal injection of LPS (1.5 mg/kg for 18h) and confirmed by iNOS expression using RT-PCR. Statistical significances ($P<0.05$) were analyzed by Student's t-test or paired t-test according to data characteristics.

The blood pressure, but not heart rate, was decreased in LPS-treated rats as compared to control rats. The contractile response to phenylephrine were dose-dependently increased from the doses of 10^{-8} M to that of 10^{-5} and were attenuated in LPS-treated rings. Both halothane and enflurane, at the doses of 1 MAC, decreased the contractile responses to phenylephrine while isoflurane did not significantly affect the contractile responses. Hydroxocobalamin (10^{-5} M) significantly potentiated the contractile responses in the LPS-treated aortic ring preparations during administration of each inhalational anesthetic or not.

From these results, it is suggested that hydroxocobalamin may improve the hemodynamics of septic patients during inhalational anesthesia.

Abbreviations: LPS, lipopolysaccharide; RT-PCR, reverse transcription-polymerase chain reaction; MAC, minimum alveolar concentration; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Key Words: Inhalational anesthetics, Sepsis, Hydroxocobalamin, Lipopolysaccharide

INTRODUCTION

Septic shock is associated with many pathophysiologic alterations, such as metabolic acidosis, potentially lethal hypotension and widespread end-organ damage. It has been reported that these changes are induced by lipopolysaccharide (LPS), which is a component of the bacterial cell walls (Morrison and Ulevitch, 1978; Raetz, 1990). Lipopolysaccharide(LPS) induces an inducible nitric oxide synthase (iNOS), which produces an enormous amount of nitric oxide, a major causative agent of sepsis.

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The inhalational anesthetics such as halothane and isoflurane have multiple effects on muscle tension and $[Ca^{2+}]_{cyt}$ and induce $[Ca^{2+}]_{cyt}$-dependent and $[Ca^{2+}]_{cyt}$-independent suppression of the contraction in vascular smooth muscle of rat aorta (Tsuchida *et al.*, 1993), which is comparable to the effect observed in cardiac myocytes (Katsuoka *et al.*, 1989).

The relaxing factors from endothelial cells such as EDRF and EDHF modulate the vascular smooth muscle tone. The endothelium-dependent vasodilation was inhibited by halothane, enflurane, and isoflurane of which isoflurane is more potent than halothane and enflurane in this regard (Uggeri, 1992). The inhibition of endothelium-dependent vasodilation by inhalational anesthetics is not primarily due to inhibition of EDRF production/release in endothelial cells, but is proximal to the site of guanylate cyclase activation in vascular smooth muscle.

Hydroxocobalamin enhanced phenylephrine-induced contractions (Martin *et al.*, 1985; Khan *et al.*, 1992) because hydroxocobalamin sequesters nitric oxide in a biological milieu by forming nitrosocobalamin and thereby inhibit the smooth muscle relaxant action of NO (Rajanayagam *et al.*, 1993). The mechanisms of action of hydroxocobalamin is, therefore, like that of hemoglobin which binds NO and thereby reduces the effects of NO and nitrovasodilators in activating guanylate cyclase (Murad *et al.*, 1978) and relaxing vascular smooth muscle (Martin *et al.*, 1985). Since hydroxocobalamin readily combines with NO to form nitrosocobalamin (Kaczka *et al.*, 1951), we suggest that hydroxocobalamin attenuate the responses to NO enormously produced in aortic rings of LPS-treated rat by sequestering NO so that the concentration of NO available for activating soluble guanylate cyclase and relaxing the smooth muscle is reduced.

In the present study, the effects of hydroxocobalamin on contractile responses to phenylephrine during the administration of inhalational anesthetics, halothane, enflurane and isoflurane, were evaluated in aortic ring preparations obtained from LPS-treated rats.

MATERIALS AND METHODS

Septic rats

Male Sprague Dawley rats weighing 250~300 g were used in this study. Septic rats were rendered by intraperitoneal injection of lipopolysaccharide (1.5 mg/kg). Eighteen hours after injection, the rats were anesthetized with sodium pentobarbital (35 mg/kg i.p.). The left carotid artery was cannulated for measuring the arterial pressure and heart rate by means of a strain gage coupler and a biotachometer coupler, respectively, connected to pressure transducer. The blood pressure and heart rate were recorded on a physiograph (Narco Bio-Systems, U.S.A.).

RT-PCR

In a separate experiment, induction of iNOS mRNA was confirmed in the aortae of LPS treated-rats using RT-PCR. To obtain a sufficient amount of RNA, the thoracic aortae of 4 rats were homogenized in 1 ml of Ultraspec™ - II RNA solution (Biotecx lab., INC.) from which total RNA was extracted and reverse transcribed (10 μg) as described before (Kim, 1996). Briefly, the first strand cDNA for iNOS was synthesized using Stratagene RT-PCR kit with an antisense primer while that for GAPDH with an oligo$(dT)_{12-18}$. PCR amplification was performed according to the following schedule: Denaturation, annealing and elongation at 94, 60 and 72℃ for 1, 1 and 1.5 minutes, respectively, for 30 cycles. To confirm the change in the iNOS mRNA level, the GAPDH mRNA level was used as a control. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. The primer sequences utilized for the experiment were as follows; iNOS sense primer (5′-ATGGCTTGCCCCTGGAAGTTTCTC-3′), antisense primer (5′-CCTCTGATGGTGCCATCGGGCATCTG-3′), GAPDH sense primer (5′-GTCATGAGCCCTTCCACGATGC-3′), antisense primer (5′-GAATCTACTGGCGTCTTCACC-3′).

Organ bath study

The thoracic aortae were excised immediately

and cleaned of fat and connective tissues on the moistened filter paper as described before (Shim, 1990). The preparations were cut into four ring segments (4 mm in length). Each aortic ring was suspended in a water-jacketed organ bath containing 20 ml of modified Krebs-bicarbonate solution of the following composition (millimolar concentrations): NaCl, 115.0 ; KCl, 4.7 ; $CaCl_2$, 2.5 ; $MgCl_2$, 1.2 ; $NaHCO_3$, 25.0 ; KH_2PO_4, 1.2 ; and dextrose, 10.0. The bathing solutions were maintained at 37±0.5℃ and aerated with a gas mixture of 95% O_2 and 5% CO_2.

Two stainless steel triangles were inserted through each vessel ring. Care was taken to avoid rubbing the endothelial surface of the vessel. One was anchored to a stationary support and the other was connected to an isometric force transducer (Grass FT03C). Aortic rings were stretched passively by imposing the optimal resting tension, 2.0 g, which was maintained throughout the experiment.

The segments were equilibrated for 120 min during which time the solution were replaced every 30 min. Thereafter, the contractile response to 50 mM KCl was elicited first to ensure stabilization of the muscles. The isometric contractions were recorded on an ink-writing physiograph (Grass model 7). After a stable plateau had developed, the rings were washed with several changes of fresh buffer. A period of 30 min was allowed for re-equilibration, after which phenylephrine were added to the organ bath in a cumulative fashion to obtain complete concentration-response relationships. After another re-equilibration time phenylephrine were added again during administraton of inhalational anesthetics. If necessary, hydroxocobalamin was pretreated 10 minutes before phenylephrine administration. The contractile responses were expressed as a percentage of maximum contraction induced by 50 mM KCl solution.

Drugs

Drugs used in the present study include ; phenylephrine HCl, hydroxocobalamin HCl (Sigma Chemical Co., U.S.A.); halothane (Il Sung Pharmaceutical Co.), enflurane (Choong Wae Pharmaceutical Co.) and isoflurane (Abbott Co.). All other reagents were analytical grade.

Statistical Analysis. The data were expressed as mean±S.E.M. and were analyzed by Student's t-test or paired t-test using SPSSWIN package. P values less than 0.05 were regarded as statistically significant.

RESULTS

Effect of LPS treatment on blood pressure and heart rate

The intraperitoneal injection of lipopolysaccharide (1.5 mg/kg), 18 hours later, significantly decreased the systolic, mean, and diastolic blood pressures without any significant change in heart rate (Table 1).

Confirmation of iNOS expression by RT-PCR

Induction of iNOS mRNA was confirmed using RT-PCR in the aortae of LPS treated-rats. To obtain adequate RNA, the thoracic aortae of 4 rats were used from which total RNA was extracted. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. To confirm the change in the iNOS mRNA level, the GAPDH mRNA level was used as a control (Fig. 1).

Table 1. Systolic, mean, and diastolic blood pressure, and heart rate in the control and LPS treated-rats

	Blood pressure			HR
	SBP	MBP	DBP	
Control	165±6.2	136±4.3	121±3.7	420±13
LPS	140±4.7*	111±5.5*	97±6.1*	425±17

P<0.05, Significantly different from control group.

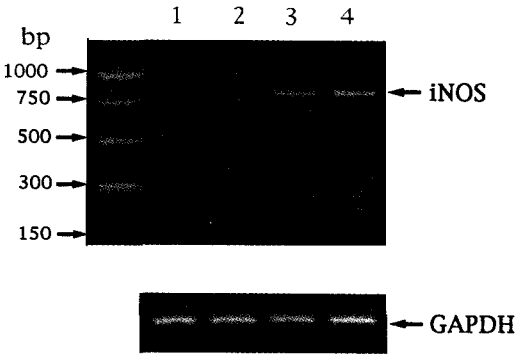

Fig. 1. Induction of iNOS mRNA in the aortae of control (lane 1 and 2) and LPS treated-rats (lane 3 and 4). To obtain the result of each lane, the aortae of 4 rats were homogenized in Ultraspec™ -II RNA solution from which total RNA was extracted and reverse transcribed (10 μg). cDNA product was amplified for 30 cycles and electrophoresed on a 1.5% agarose gel containing ethidium bromide. GAPDH was used as control.

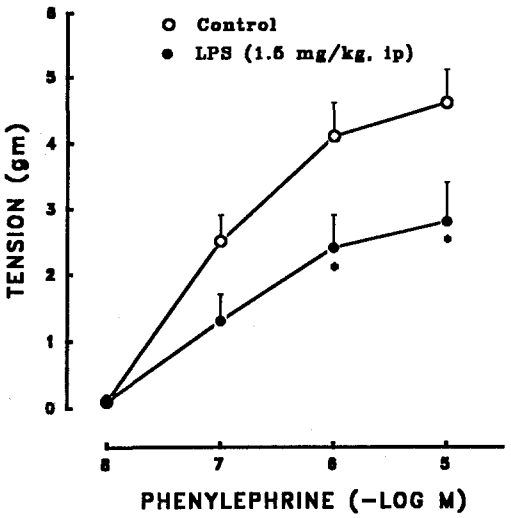

Fig. 2. Cumulative log concentration-response curves for the contractile responses to phenylephrine in the aortic rings of control (○) and LPS-treated (●) rats. The contractile responses were expressed as gm tension. The data were expressed as means of 7 experiments with vertical bars showing S.E.M. *Significantly different from control ($P<0.05$).

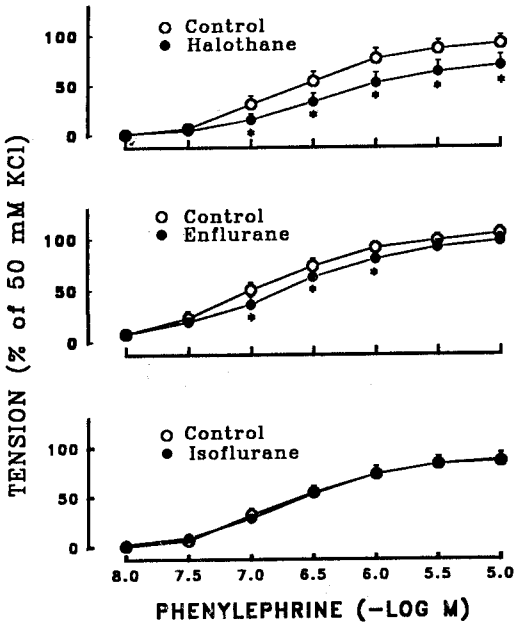

Fig. 3. Cumulative log concentration-response curves for the contractile responses to phenylephrine in the aortic rings of LPS-treated rats during administration of each inhalational anesthetic (●) or not (○). The contractile responses were expressed as a percentage of the maximum contraction developed by 50 mM KCl. The data were expressed as means of 7 experiments with vertical bars showing S.E.M. * Significantly different from control ($P<0.05$).

Effect of LPS treatment on contractile response to phenylephrine

The contractile response to phenylephrine were dose-dependently increased from the doses of 10^{-8} M to that of 10^{-5} and were attenuated in LPS-treated rings (Fig. 2).

Effect of inhalational anesthetics on contractile response to phenylephrine

Both halothane and enflurane, at the doses of 1 MAC (0.75 and 1.68%, respectively), decreased the contractile responses to phenylephrine while isoflurane (1 MAC, 1.40%) did not signifi-

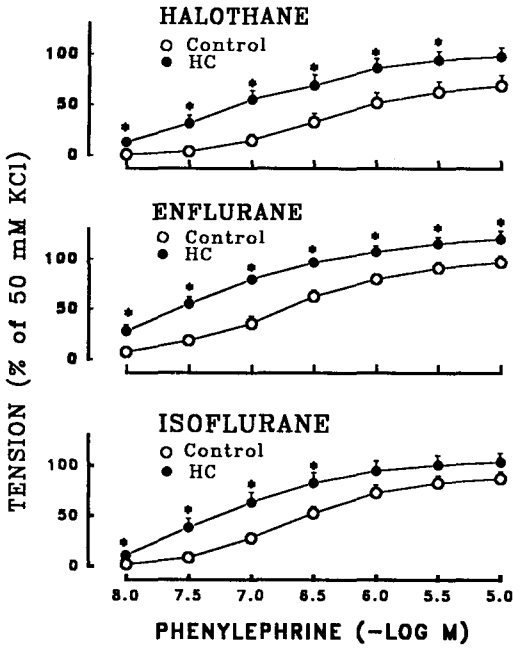

Fig. 4. Cumulative log concentration-response curves for the contractile responses to phenylephrine in the aortic rings of LPS-treated rats during administration of each inhalational anesthetic in the presence (●) or absence (○) of hydroxocobalamin (HC). The contractile responses were expressed as a percentage of the maximum contraction developed by 50 mM KCl. The data were expressed as means of 7 experiments with vertical bars showing S.E.M. * Significantly different from control ($P<0.05$).

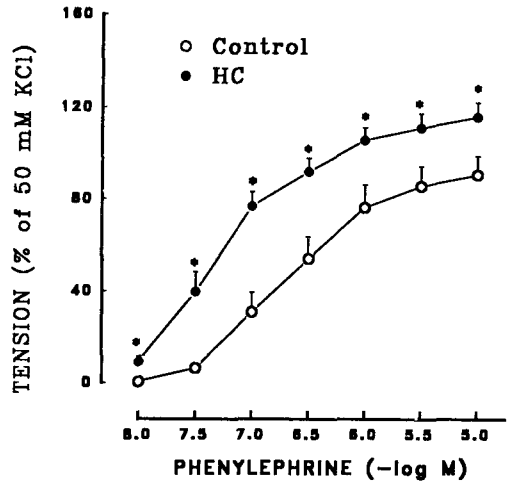

Fig. 5. Cumulative log concentration-response curves for the contractile responses to phenylephrine in the aortic rings of LPS-treated rats in the presence (●) or absence (○) of hydroxocobalamin (HC). The contractile responses were expressed as a percentage of the maximum contraction developed by 50 mM KCl. The data were expressed as means of 7 experiments with vertical bars showing S.E.M. * Significantly different from control ($P<0.05$).

cantly affect the contractile responses (Fig. 3).

Effect of hydroxocobalamin on contractile response to phenylephrine

Hydroxocobalamin (10^{-5} M) significantly potentiated the contractile responses in the LPS-treated aortic ring preparations during administration of each inhalational anesthetic or not (Fig. 4, 5).

DISCUSSION

The intraperitoneal injection of lipopolysaccharide (1.5 mg/kg) effectively developed sepsis 18 hours later which significantly decreased the systolic, mean, and diastolic blood pressures without any significant change in heart rate (Table 1). Induction of iNOS mRNA in the aortae of LPS treated-rats was also confirmed using RT-PCR (Fig. 1).

The contractile response to phenylephrine were dose-dependently increased from the doses of 10^{-8} M to that of 10^{-5} and were attenuated in LPS-treated rings (Fig. 2). A high K^+ solution depolarizes cell membrane and opens voltage-dependent Ca^{2+} channels in vascular smooth muscle, resulting in increases in $[Ca^{2+}]_{cyt}$ and muscle tension (Haeusler, 1983). Vasoconstrictors such as norepinephrine and phenylephrine induce muscle contraction depending on, not only the influx of Ca^{2+} through the sarcolemma and release of Ca^{2+} from the sarcoplas-

mic reticulum, but also the increase in Ca^{2+} sensitivity of the contractile elements (Sato *et al.*, 1988). Therefore, these agents could induce a greater contraction than high K^+ for a given increase in $[Ca^{2+}]_{cyt}$. A norepinephrine-induced increase in Ca^{2+} sensitivity was is shown in the experiment in which there was a sustained increase in muscle contraction after the $[Ca^{2+}]_{cyt}$ returned to its resting level (Tsuchida *et al.*, 1993). As a consequence of increasing Ca^{2+} sensitivity, norepinephrine-induced contractions are less sensitive to Ca^{2+} channel blockade than are contractions induced by increased K^+.

Both halothane and enflurane, at the doses of 1 MAC, decreased the contractile responses to phenylephrine while isoflurane did not significantly affect the contractile responses (Fig. 3). The inhalational anesthetics such as halothane and isoflurane have multiple effects on muscle tension and $[Ca^{2+}]_{cyt}$ and induce $[Ca^{2+}]_{cyt}$-dependent and $[Ca^{2+}]_{cyt}$-independent suppression of the contraction in vascular smooth muscle of rat aorta (Tsuchida *et al.*, 1993), which is comparable to the effect observed in cardiac myocytes (Katsuoka *et al.*, 1989). These anesthetic agents, during the resting state, increase $[Ca^{2+}]_{cyt}$ which is released mainly from the intracellular Ca^{2+} storage site, but with only slight effects on resting tension except for 3% halothane exposure in which an apparent but transient increase in muscle tension was observed. However, they attenuate high K^+- and norepinephrine-induced increases in muscle tension and $[Ca^{2+}]_{cyt}$ in a concentration-dependent manner (Tsuchida *et al.*, 1993). An increase in $[Ca^{2+}]_{cyt}$ does not always elicit muscle contraction in the vascular smooth muscle. It is, therefore, possible that the halothane-induced increase in $[Ca^{2+}]_{cyt}$ might not have reached the threshold concentration that evokes muscle contraction and/or that halothane-induced relaxation mechanism(s) might have been activated.

Sprague *et al.* (1974) reported that halothane and isoflurane stimulate the formation of cyclic adenosine monophosphate in the vascular smooth muscle of rat aorta. Cyclic adenosine monophosphate is known to decrease not only $[Ca^{2+}]_{cyt}$ (Itoh *et al.*, 1982; Meisheri and Breemen, 1982) but also the sensitivity of contractile elements to Ca^{2+} by phosphorylating myosin light chain kinase (Adelstein *et al.*, 1978). Therefore, anesthetic-induced increases in cyclic adenosine monophosphate in smooth muscle might be involved in the anesthetic-induced relaxation mechanism. Another possibility in regard to the relaxation mechanism has been indicated in a report in which halothane induced a decrease in membrane-bound Ca^{2+}/phospholipid-dependent protein kinase in canine tracheal smooth muscle during carbachol contraction (Yamakage, 1992).

Similarly to the effect of a Ca^{2+} channel blocker, the anesthetic agents were likely to suppress increases in muscle tension and $[Ca^{2+}]_{cyt}$ more during high K^+-induced contraction than those during norepinephrine-induced contraction. The halothane-induced relaxation was greater than the effect of isoflurane administered during phenylephrine-induced contraction in the vascular smooth muscle of rat aorta (Sprague *et al.*, 1974).

An interesting possibility is that the relaxing factors from endothelial cells such as EDRF and EDHF modulate the vascular smooth muscle tone. The endothelium-dependent vasodilation was inhibited by halothane, enflurane, and isoflurane of which isoflurane is more potent than halothane and enflurane in this regard (Uggeri, 1992). The inhibition of endothelium-dependent vasodilation by inhalational anesthetics is not primarily due to inhibition of EDRF production/release in endothelial cells, but is proximal to the site of guanylate cyclase activation in vascular smooth muscle. Further study would be necessary to elucidate the mechanism underlying this.

Hydroxocobalamin (10^{-5} M) significantly potentiated the contractile responses in the LPS-treated aortic ring preparations during administration of each inhalational anesthetic or not (Fig. 4, 5). Phenylephrine-induced contractions were enhanced by hydroxocobalamin, and this effect has also been reported with haemoglobin (Martin *et al.*, 1985; Khan *et al.*, 1992). Hydroxocobalamin sequesters nitric oxide in a biological milieu by forming nitrosocobalamin and thereby inhibit the smooth muscle relaxant action of NO (Rajanayagam *et al.*, 1993). Hydroxocobalamin resembled hemoglobin in

that it too reduced the endothelium-dependent relaxant action of acetylcholine in rat aortic rings

The postulated mechanisms of action of hydroxocobalamin is, therefore, like that of hemoglobin which binds NO and thereby reduces the effects of NO and nitrovasodilators in activating guanylate cyclase (Murad *et al.*, 1978) and relaxing vascular smooth muscle (Martin *et al.*, 1985). Since nitrosocobalamin did not produce relaxation of aortic rigs, the NO bound in it is not available for activation of the soluble guanylate cyclase in the vascular smooth muscle.

Haemoglobin inhibits endothelium-dependent relaxations in vascular preparations (Martin *et al.*; 1985 ; Ignarro *et al.*, 1987). Likewise, hydroxocobalamin inhibited, the endothelium-dependent relaxant action of ACh in aortic rings and, as was reported for haemoglobin (Martin *et al.*, 1985), hydroxocobalamin was more effective in reducing endothelium-dependent that NO-induced relaxations. A possible explanation for this difference is that the total amount of NO generated from ACh-activated endothelial cells is likely to be considerably less than the amount of exogenous NO producing an equivalent relaxation ; hence, a given amount of hydroxocobalamin or haemoglobin in the organ bath would more completely mop up the endothelium-derived NO. From these results, it is plausible that hydroxocobalamin may be used to improve the hemodynamics of septic patients during inhalational anesthesia.

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=국문초록=

흡입마취제 투여시 내독소혈증흰쥐 대동맥 수축반응에 미치는 Hydroxocobalamin의 효과

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패혈증 환자에 흡입마취제를 투여하는 것은 혈역동학에 심각한 영향을 초래할 수 있다. 흡입마취제 투여시 내독소혈증흰쥐 대동맥 수축반응에 미치는 hydroxocobalamin의 영향을 알아보기 위해, 내독소 (1.5 mg/kg)를 복강내 투여한 뒤 18시간 후에 대동맥을 적출하여 phenylephrine에 대한 수축반응과 이에 대한 흡입마취제 및 hydroxocobalamin의 작용을 알아보았다.

패혈증이 적절히 유발되었는 지를 확인하기 위해 RT-PCR을 이용하여 혈관 평활근에서 iNOS 유도를 확인하였다. 내독소 처치에 의해 수축기 및 확장기 혈압이 대조군에 비해 유의하게 감소되었으나, 심박동수는 영향이 없었다. Phenylephrine에 대한 수축반응은 10^{-8} M부터 10^{-5} M까지 용량 의존적으로 증가했으며, 내독소혈증흰쥐 대동맥에서 수축반응이 억제되었다. halothane과 enflurane은 1 MAC 농도에서 phenylephrine에 의한 수축반응을 유의하게 억제했으나 isoflurane은 영향이 없었다. Hydroxocobalamin (10^{-5} M)은 흡입마취제의 종류 및 투여 유무에 관계없이 내독소혈증흰쥐 대동맥 수축반응을 증가시켰다.

이상의 결과로 미루어보아 hydroxocobalamin은 패혈증 환자의 흡입마취시 혈역동을 개선시킬 것으로 사료된다.