N\textsuperscript{G}-Nitro-L-arginine Methyl Ester, but Not Methylene Blue, Attenuates Anaphylactic Hypotension in Anesthetized Mice

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Received January 19, 2007; Accepted May 1, 2007

Abstract. To clarify the role of NO in mouse anaphylactic hypotension, effects of a nitric oxide (NO) synthase inhibitor, \textsuperscript{N}\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), on antigen-induced hypotension and portal hypertension were determined in anesthetized BALB/c mice. Systemic arterial pressure (Psa), central venous pressure (Pcv), and portal venous pressure (Ppv) were directly and simultaneously measured. Mice were first sensitized with ovalbumin, and then the injection of antigen was used to decrease Psa and increase Ppv. Pretreatment with L-NAME (1 mg/kg) attenuated this antigen-induced systemic hypotension, but not the increase in Ppv. The effect of inhibitors of soluble guanylate cyclase on anaphylactic hypotension were studied with either methylene blue (3.0 mg/kg) or 1H-[1,2,4]oxadiazole[4,3-\textalpha]quinoxalin-1-one (10 mg/kg). Neither modulated any antigen-induced changes. Furthermore, methylene blue did not improve systemic hypotension induced by Compound 48/80 (4.5 mg/kg), a mast cell degranulator, which can produce non-immunological anaphylactoid reactions. These data show in anesthetized BALB/c mice that L-NAME attenuated anaphylactic hypotension without affecting portal hypertension. This beneficial effect of L-NAME appears not to depend on the soluble guanylate cyclase pathway.

Keywords: anaphylactic shock, portal venous pressure, \textsuperscript{N}\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), methylene blue

Introduction

Anaphylactic hypotension is sometimes fatal (1) and is primarily caused by a decreased blood flow to the heart because left ventricular function is relatively well preserved during anaphylactic shock (2). However, hemodynamic mechanisms responsible for anaphylactic hypotension are not fully understood. We have proposed that the liver and splanchnic vascular beds are involved in anaphylactic hypotension (3–6). Indeed, the liver plays a crucial role in the pathogenesis of circulatory collapse in canine anaphylactic shock (3, 7). Anaphylaxis-induced hepatic venous constriction induces pooling of blood in the liver, as well as in upstream splanchnic organs. Both effects reduce venous return with a resultant decrease in stroke volume and systemic arterial pressure. In addition, in sensitized rabbits, anaphylactic hypotension is accompanied by substantial portal hypertension (4), although right heart overload due to pulmonary hypertension is recognized as a causative factor (8). In rats, antigen-induced hepatic venoconstriction also contributes to anaphylactic hypotension (5), and we have recently reported that the liver and splanchnic vascular beds are also involved in mouse anaphylactic hypotension (6).

Nitric oxide (NO) has been shown to play a primary and harmful role in circulatory shock, causing progressive refractory hypotension which ultimately leads to multiple organ dysfunction and death (9–11). Although mice have been frequently used for a variety of physiological studies because of development of genetic engineering, few studies have examined a possible role for NO in mouse anaphylactic hypotension (10, 12). Furthermore, it is not known how NO affects anaphylactic hepatic venoconstriction in mice.

The objective of this study was to investigate the role of NO on both systemic and hepatic circulation during anaphylactic hypotension in anesthetized mice.
To achieve this, systemic arterial pressure and portal venous pressure were directly measured in sensitized mice intravenously administered ovalbumin antigen. In addition, we determined the effects of methylene blue or 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), both inhibitors of soluble guanylate cyclase, on anaphylactic hypotension to define any role for activation of guanylate cyclase and subsequent generation of cGMP in the vasorelaxant action of NO (13). A key reason for studying the effect of methylene blue in mouse anaphylactic hypotension in the present study is the suggestion that methylene blue might be a potential therapeutic drug in anaphylactic shock (14).

Materials and Methods

Animals

The experiments conducted in the present study were approved by the Animal Research Committee of Kanazawa Medical University. We used 57 male BALB/c mice (SLC, Shizuoka) weighing 27 ± 3 g in this study. Mice were maintained at 23°C and under pathogen-free conditions on a 12:12-h dark/light cycle, with food and water ad libitum.

Sensitization

Mice were actively sensitized by the subcutaneous injection of an emulsion made by mixing aluminum potassium sulfate adjuvant (2 mg) with 0.01 mg ovalbumin (grade V; Sigma Chemical Co., St. Louis, MO, USA) dissolved in physiological saline (0.2 ml). The antigen emulsion was injected again one week after the first antigen injection. Non-sensitized (control) mice were injected with aluminum potassium sulfate adjuvant and ovalbumin-free saline. One week after the second injection, the mice were used for the following experiments except the experiment for compound 48/80 (C48/80), a mast cell degranulating agent.

Protocol

Mice were anesthetized with pentobarbital sodium (90 mg/kg, i.p.) and placed on a thermostatically controlled heating pad (ATC-101B; Unique Medical, Tokyo) to maintain body temperature at 37 ± 0.2°C throughout the experiment. The adequacy of anesthesia was monitored by the stability of blood pressure and respiration under control conditions and during tail pinch. Supplemental doses of anesthetic (10% of initial dose) were given intraperitoneally if necessary. To ensure airway patenty, tracheotomy followed by insertion of a tracheal tube (18G stainless steel needle) was performed. The right femoral artery was catheterized to measure systemic arterial pressure (Psa) with a transducer (TP-400T; Nihon Kohden, Tokyo). The right external jugular vein was catheterized, and the catheter tip was positioned at the confluence of the superior vena cava and the right atrium to measure the central venous pressure (Pcv). Following a laparotomy, a catheter (ID 0.47 mm, OD 0.67 mm) was inserted into the main portal vein for continuous measurement of portal venous pressure (Ppv). The Psa, Pcv, and Ppv were continuously measured and continuously displayed on a thermal physiograph (RMP-6008, Nihon-Kohden). Outputs were also digitally recorded at 20 Hz (PowerLab; AD Instruments, Sydney, Australia).

The following three experimental protocols were employed:

1) The effect of an NO synthesis inhibitor, L-NAME, on anaphylactic hypotension: either L-NAME (1.0 mg/kg, 25 µl; n = 8) or Nω-nitro-L-arginine methyl ester (D-NAME) (1.0 mg/kg, 25 µl; n = 8) was intravenously administered. After 10 min, 0.01 mg of ovalbumin antigen (in 100 µl saline) was intravenously administered.

2) The effect of inhibitors of soluble guanylate cyclase, methylene blue or ODQ, on anaphylactic hypotension: either methylene blue (3.0 mg/kg, 25 µl; n = 7) or saline (25 µl, n = 5) was intravenously administered into sensitized mice. After 2 min, the ovalbumin antigen was administered as above. In 6 mice, ODQ (10 mg/kg in 50 µl DMSO) was intraperitoneally administered 1.5 h prior to the injection of ovalbumin antigen.

3) The effect of methylene blue on anaphylactoid reactions induced by C48/80: either methylene blue (3.0 mg/kg; 25 µl, n = 5) or saline (25 µl, n = 5) was intravenously administered into non-sensitized mice. After 2 min, C48/80 (4.0 mg/kg, 100 µl) was intravenously administered.

The following drugs were used: methylene blue (Waldech GmbH & Co., Muenster, Germany); L-NAME, D-NAME, ODQ, and C48/80 (Sigma).

Statistics

All results are expressed as the means ± S.D. Statistical analysis was performed by repeated measures analysis of variance. Comparison of individual points within groups was made by analysis of variance followed by the Bonferroni post-test correction method. Comparison of individual points between two groups and among four groups was made by Student’s t-test and analysis of variance followed by the Bonferroni post-test correction method, respectively. Differences were considered statistically significant at P<0.05.
Results

Intravenous injection of L-NAME, an inhibitor of NO synthase, significantly increased Psa from a baseline of 99 ± 6 to 116 ± 6 mmHg at 10 min, while injection of D-NAME, an inactive isomer of L-NAME, did not change Psa. At 10 min after injection of either L-NAME or D-NAME, ovalbumin antigen was injected to induce anaphylactic hypotension. Figure 1 shows representative examples of the response to intravenous injection of ovalbumin antigen in sensitized mice pretreated with L-NAME or D-NAME. Figure 2 shows the summary data for the time course of changes in Psa, Ppv, and Pcv. After an antigen injection, Psa and Ppv increased in the D-NAME group. Psa increased to the peak of 107 ± 5 mmHg initially and then progressively and significantly decreased to 50 ± 8 mmHg. Ppv increased from the baseline of 5.6 ± 0.7 cmH₂O to the peak of 9.8 ± 0.8 cmH₂O in the D-NAME groups. In the L-NAME group, after antigen, Psa increased to a peak of 135 ± 7 mmHg and then declined but not significantly from baseline until 9, 10, and 20 min. Significant differences in Psa between the L-NAME and D-NAME group were observed up to 50 min after antigen. In contrast to the Psa response, Ppv in the L-NAME group increased in a similar way to the D-NAME group: Ppv increased from the baseline of 5.6 ± 0.7 cmH₂O to a peak of 9.9 ± 0.8 cmH₂O after antigen. Pcv did not significantly change after antigen in either the L-NAME or D-NAME group.

To examine how NO may attenuate anaphylactic hypotension, we used pretreatment with methylene blue in anesthetized mice sensitized with ovalbumin. Intravenous injection of methylene blue did not change the baseline values of Psa, Ppv, and Pcv. After 2 min, antigen was injected to evoke anaphylactic hypotension. Figure 3 shows anaphylactic hypotension in the presence of methylene blue. Pretreatment with methylene blue

Fig. 2. The summary of changes in the systemic arterial pressure, portal venous pressure, and central venous pressure after an ovalbumin antigen injection in the presence of D-NAME or L-NAME. Open circle, D-NAME control (non-sensitized) (n = 4); closed circle, D-NAME anaphylaxis (n = 8); open square, L-NAME control (non-sensitized) (n = 4); closed square, L-NAME anaphylaxis (n = 8). Values are means ± S.D.; *P<0.05 vs the baseline values, **P<0.05 vs the D-NAME anaphylaxis group.

Fig. 1. Representative recordings of the response to ovalbumin antigen of an anesthetized mouse pretreated with D-NAME (A) and L-NAME (B). The arrow on each trace indicates the antigen injection.
did not significantly affect the antigen-induced changes in Psa, Ppv, or Pcv. In addition, use of ODQ (10 mg/kg), more specific soluble guanylate cyclase inhibitor, also failed to influence the anaphylactic response.

The fact that methylene blue had no effect on mouse anaphylactic hypotension was unexpected, because Buzato et al. (15) reported that methylene blue improved systemic hypotension induced by C48/80, a mast cell degranulator in the rabbit. So the effect of methylene blue on systemic hypotension induced by C48/80 was also examined in non-sensitized BALB/c mice. Figure 4 shows the summary data for time course changes in Psa, Ppv, and Pcv after an intravenous injection of C48/80 in non-sensitized mice pretreated with or without methylene blue. In control mice, Psa initially increased to 114 ± 15 mmHg from a baseline of 90 ± 8 mmHg after C48/80. Thereafter, Psa dropped to 58 ± 2 mmHg at 8 min and then gradually recovered. Ppv was modestly increased, from a baseline of 5.9 ± 0.5 to 8.3 ± 0.6 cmH2O at 2 min after an injection of C48/80. Ppv then returned to the baseline levels by 8 min. Pcv was only slightly decreased after C48/80. Pretreatment with methylene blue did not significantly attenuate the C48/80-induced decrease in Psa (Fig. 4), and likewise, there were no substantial differences in Ppv or Pcv to C48/80 with methylene blue (Fig. 4).

**Discussion**

The present study showed the NO synthesis inhibitor, L-NAME, attenuated anaphylactic systemic hypotension, but not anaphylactic portal hypertension, in anesthetized BALB/c mice. Although attenuation of
anaphylactic hypotension with L-NAME has been reported previously (10, 12), the present study reports the new observation that L-NAME does not affect anaphylactic portal hypertension. Furthermore, use of the soluble guanylate cyclase inhibitor methylene blue unexpectedly failed to have any effect against anaphylactic hypotension in the mouse. Methylene blue also failed to improve systemic hypotension induced by C48/80, a mast cell degranulator, that caused a systemic hypotension similar to anaphylaxis.

Anaphylactic hypotension is primarily caused by a decrease in blood flow to the heart (2). An increased resistance to venous return is important in the pathogenesis of circulatory collapse in anaphylactic shock (3). We have previously reported that anaphylaxis-induced increase in venous resistance is in part caused by hepatic vasoconstriction in dogs (7), guinea pigs (16), rats (5), and rabbits (4). We speculate that anaphylaxis causes hepatic vasoconstriction and portal hypertension, resulting in congestion of the upstream splanchnic organs, with a resultant decrease in venous return and effective circulating blood volume, and finally augmentation of anaphylactic hypotension. Indeed, elimination of the liver and splanchnic circulation by total hepatectomy combined with ligation of the celiac and mesenteric arteries attenuates anaphylactic hypotension in anesthetized rats (5). More recently, the same surgical procedures (hepatectomy and elimination of the splanchnic vascular bed) have been shown to attenuate mouse anaphylactic hypotension (6). However, in the present study, antigen-induced increases in Ppv were observed in both L-NAME and D-NAME groups, although a significantly smaller decrease in Psa after antigen occurred in the L-NAME group compared to the D-NAME group. This suggests that L-NAME does not affect anaphylactic hepatic vasoconstriction and that the beneficial anti-hypotensive effect of L-NAME may be exerted not on the hepatic vessels but at extrahepatic sites, possibly systemic arterioles. In this regard, we recently showed that mouse hepatic vessels display weak responses to L-NAME: L-NAME did not increase the basal Ppv of hepatic vascular tone in isolated perfused mouse liver nor did it augment hepatic vasoconstriction to either the anaphylaxis-related mediator of platelet-activating factor (PAF) (17) or thromboxane A2 (unpublished observation).

NO relaxes vascular smooth muscle cells mainly through activation of soluble guanylate cyclase (sGC) and subsequent cyclic GMP-dependent modification of several intracellular processes, including the phosphorylation of proteins of the contractile apparatus and of pumps or channels involved in modulating intracellular calcium levels (13). Recent studies by Evora’s group reported that one inhibitor of sGC, methylene blue, can reverse clinical anaphylactic shock induced by injected contrast media (12) and prolong the survival of rabbits during experimental anaphylaxis (15). However, in the present study, methylene blue did not prevent anaphylactic hypotension to the same extent. Furthermore, we could not find any beneficial effects of methylene blue against C48/80-induced hypotension, as reported by Buzato et al. (15), in anesthetized mice. In addition, we also failed to alter the anaphylactic responses with another sGC inhibitor, ODQ. The reason for this disparity is not clear, but it might possibly reflect a species difference. In agreement with our data, Cauwels et al. showed that ODQ did not protect against shock induced by PAF in the mouse and that deficiency of NO synthase inhibition on anaphylactic hypotension. Anaphylactic hypotension to the same extent. Furthermore, pretreatment with either methylene blue or ODQ did not improve systemic hypotension induced by antigen. Thus, it is suggested that sGC independent events downstream from the NO produced during anaphylaxis in the mouse underlie the beneficial action of NO synthase inhibition on anaphylactic hypotension.

**Acknowledgments**

We thank Professor Chris J Garland and Dr. Kim A Dora for carefully reading a draft version of the manuscript and suggesting many improvements. This study was supported by a Grant for Promoted Research from Kanazawa Medical University (S2006-8), Collaborative Research from Kanazawa Medical University (C2005-1), and a Grant-in-Aid for Scientific Research (18591730) from the Ministry of Education, Culture, Sports, Sciences, and Technology of Japan.
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