

***N*^G-NITRO-L-ARGININE METHYL ESTER POTENTIATES ANAPHYLACTIC VENOCONSTRICTION IN RAT PERFUSED LIVERS**

**Toshishige Shibamoto,* Takaharu Ishibashi,[†] Tomohiro Shimo,[‡] Sen Cui,* Hiromichi Takano,*
Hideaki Tsuchida,[‡] Matomo Nishio[†] and Yasutaka Kurata***

*Departments of *Physiology, [†]Pharmacology and [‡]Anaesthesiology, Kanazawa Medical University, Uchinada, Japan*

SUMMARY

1. The effects of the nitric oxide (NO) synthase inhibitor *N*^G-nitro-L-arginine methyl ester (L-NAME) on anaphylaxis-induced venoconstriction were examined in rat isolated livers perfused with blood-free solutions in order to clarify the role of NO in anaphylactic venoconstriction.

2. Rats were sensitized with ovalbumin (1 mg) and, 2 weeks later, livers were excised and perfused portally in a recirculating manner at a constant flow with Krebs–Henseleit solution. The antigen (ovalbumin; 0.1 mg) was injected into the reservoir 10 min after pretreatment with L-NAME (100 µmol/L) or D-NAME (100 µmol/L) and changes in portal vein pressure (Ppv), hepatic vein pressure (Phv) and perfusate flow were monitored. In addition, concentrations of the stable metabolites of NO (NO₂⁻ and NO₃⁻) were determined in the perfusate using an HPLC–Griess system.

3. The antigen caused hepatic venoconstriction, as evidenced by an increase in Ppv from a mean (±SEM) baseline value of 7.7 ± 0.1 cmH₂O to a peak of 21.4 ± 1.1 cmH₂O at 3 min in D-NAME-pretreated livers. Pretreatment with L-NAME augmented anaphylactic venoconstriction, as reflected by a higher Ppv (27.4 ± 0.8 cmH₂O) after antigen than observed following D-NAME pretreatment. The addition of L-arginine, a precursor for the synthesis of NO, reversed the augmentation of anaphylactic venoconstriction by L-NAME. This suggests that hepatic anaphylaxis increased the production of NO, which consequently attenuated anaphylactic venoconstriction. However, perfusate NO_x levels did not increase significantly after antigen in livers pretreated with either L-NAME or D-NAME.

4. In conclusion, L-NAME potentiates rat anaphylactic hepatic venoconstriction, suggesting that NO contributes to the attenuation of the venoconstriction. However, this functional evidence was not accompanied by corresponding changes in perfusate NO_x concentrations.

Key words: anaphylactic shock, HPLC–Griess system, isolated perfused rat liver, nitric oxide, NO_x.

INTRODUCTION

Anaphylactic hypotension is sometimes life threatening and is caused by a decrease in effective circulating blood volume.¹ Nitric oxide (NO), which is produced in endothelial cells in response to either hormonal or physical stimuli, such as shear stress, regulates the vascular system.^{2,3} Nitric oxide seems to play an important pathophysiological role in modulating the systemic changes associated with anaphylaxis. An NO synthase (NOS) inhibitor was shown to attenuate hypotension and haemoconcentration and decrease venous return in this condition, although it did not improve cardiac depression.⁴ Conversely, NO functionally antagonizes the effects of vasoconstrictors released during anaphylaxis and there is experimental evidence that NO may reduce some pathophysiological changes associated with anaphylaxis, except for vasodilatation.⁴

It is well known that vasoconstriction of the liver contributes to the control of systemic circulation: passive blood mobilization to and from the liver, which is caused by hepatic vasoconstriction, influences venous return to the heart and, hence, systemic circulation.⁵ Indeed, it has been reported that hepatic venoconstriction is involved, in part, in anaphylactic hypotension.^{6,7} Anaphylaxis causes hepatic venoconstriction, as observed in rats,^{7,8} guinea-pigs⁹ and dogs,^{10,11} resulting in portal hypertension that then causes congestion of the upstream splanchnic organs, with a resultant decrease in venous return and effective circulating blood volume and finally augmentation of anaphylactic hypotension. We have shown recently that hepatic venoconstriction is observed in response to antigen in sensitized guinea-pig livers and that NO attenuates constriction of these vessels.⁹ Anaphylactic hepatic venoconstriction, based on an increase in portal venous pressure, was also observed in rats.^{7,8} However, it is not known whether NO can be released in rat liver vasculature exposed to anaphylaxis and can modulate anaphylactic hepatic venoconstriction.

Thus, to investigate these issues, anaphylactic venoconstriction was examined with rat isolated livers that were perfused with a blood-free solution under constant flow in the presence of the NOS inhibitor *N*^G-nitro-L-arginine methyl ester (L-NAME) or D-NAME, an inactive enantiomer of L-NAME. The effect of L-NAME was further examined with simultaneous pretreatment with L-arginine. In addition to this functional study, we attempted to provide physical evidence by measuring perfusate NO₂⁻ and NO₃⁻ (stable metabolites of NO) with the sophisticated HPLC–Griess system (ENO-10) to elucidate the contribution of NO to functional effects observed.

Correspondence: Dr Toshishige Shibamoto, Department of Physiology, Kanazawa Medical University, Daigaku 1-1, Uchinada, Ishikawa 920-0293, Japan. Email: shibamo@kanazawa-med.ac.jp

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METHODS

Animals

Twenty-three male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 333 ± 4 g were used in the present study. Rats were maintained under pathogen-free conditions on a 12 h dark–light cycle and allowed food and water *ad libitum*. The experiments conducted in the present study were approved by the Animal Research Committee of Kanazawa Medical University. Rats were actively sensitized by the subcutaneous injection of an emulsion made by mixing equal volumes of complete Freund's adjuvant (0.5 mL) with 1 mg ovalbumin (grade V; Sigma, St Louis, MO, USA) dissolved in physiological saline (0.5 mL).

Isolated perfused liver

Two weeks after sensitization, animals were anaesthetized with pentobarbital sodium (70 mg/kg, i.p.) and ventilated mechanically with room air. The basic methods for rat isolated perfused livers have been described elsewhere.⁷ Briefly, a catheter was placed in the right carotid artery for the injection of heparin. After laparotomy, the hepatic artery was ligated. After intra-arterial heparinization (500 U/kg), the intra-abdominal inferior vena cava (IVC) above the renal veins was ligated and the portal vein was cannulated with a stainless-steel cannula (1.3 mm ID, 2.1 mm OD) for portal perfusion. After thoracotomy, the supradiaphragmatic IVC was cannulated through an incision in the right atrium with a large stainless-steel cannula (2.1 mm ID, 3.0 mm OD). Then, portal perfusion was started at a constant flow rate (43 mL/min) with Krebs–Henseleit solution (composition (in mmol/L): NaCl 118; KCl 5.9; MgSO₄ 1.2; CaCl₂ 2.5; NaH₂PO₄ 1.2; NaHCO₃ 25.5; glucose 5.6). Initially, livers were perfused in a non-recirculating manner with perfusate that was pumped using a Masterflex pump (Cole-Parmer, Chicago, IL, USA) from the venous reservoir through a heat exchanger (37°C). When the colour of the effluent became translucent, the perfusion mode was changed to recirculating. The recirculating perfusate volume was 40 mL. The perfusate was oxygenated in the venous reservoir by continuous bubbling with 95% O₂ and 5% CO₂. The portal venous (Ppv) and hepatic venous (Phv) pressures were measured with pressure transducers (TP-400T; Nihon-Kohden, Tokyo, Japan) attached by a sidearm to the appropriate cannulas with the reference points at the hepatic hilus. Portal perfusate flow rate (Qpv) was measured with an electromagnetic flow meter (MFV 1200; Nihon-Kohden) and the flow probe was positioned in the inflow line. The Ppv, Phv and Qpv were monitored continuously and displayed through a thermal physiograph (RMP-6008; Nihon-Kohden). Outputs were also digitized by the analogue–digital converter at a sampling rate of 100 Hz using a personal computer.⁷

Hepatic haemodynamic parameters were observed for at least 20 min after the start of perfusion. Then, the livers were divided into three groups: (i) D-NAME ($n = 9$), in which 100 μ mol/L D-NAME (Sigma) was administered into the reservoir; (ii) L-NAME ($n = 9$), in which 100 μ mol/L L-NAME (Sigma) was administered into the reservoir; and (iii) L-arginine + L-NAME ($n = 5$), in which L-arginine (1 mmol/L; Sigma) plus L-NAME (100 μ mol/L) were administered into the reservoir. Thus, any livers studied were pretreated with either L-NAME or D-NAME. Ovalbumin (0.1 mg) was injected into the reservoir 10 min after the injection of L-NAME or D-NAME. In each experimental group, measurements were performed up to 30 min after the administration of antigen. The total portal–hepatic venous (Rt) resistance was calculated using the formula:

$$Rt = (Ppv - Phv)/Qpv$$

Measurement of NO_x

Portal (inflow) and hepatic venous (outflow) perfusate samples (0.5 mL) were simultaneously and instantaneously obtained from the inflow and outflow lines, respectively, before injection of L- or D-NAME just before antigen injection (baseline) and 0.5, 1, 3 and 6 min after antigen injection. NO₂⁻ and NO₃⁻ were separated and measured using the HPLC–Griess system (ENO-10; Eicom, Kyoto, Japan). The detection limit and the sensitivity for NO₃⁻ were 10 nmol/L

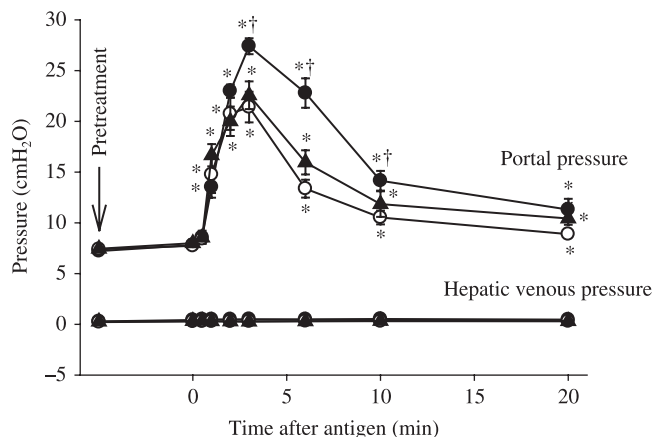


Fig. 1 Summary of changes in portal venous pressure and hepatic venous pressure after antigen injection in *N*^G-nitro-D-arginine methyl ester (D-NAME; $n = 9$; ○), *N*^G-nitro-L-arginine methyl ester (L-NAME; $n = 9$; ●) and L-arginine + L-NAME ($n = 5$; ▲) groups. Data are the mean $\pm$ SEM. * $P < 0.05$ compared with baseline; † $P < 0.05$ compared with the D-NAME and L-arginine + L-NAME groups.

under routine setup, whereas under the highly sensitive mode for NO₂⁻ (the reduction column was omitted and 100 μ L sample was loaded without deproteinization), the detection limit was 2 nmol/L and the sensitivity was 1–2 nmol/L. The inter- and intra-assay coefficients of variation were below 0.5% for NO₃⁻ and approximately 5% for NO₂⁻ for highly sensitive quantification.^{12,13} Special attention was paid to avoid any contamination with NO_x throughout the entire procedure.¹⁴

Statistics

All results are expressed as the mean $\pm$ SEM. Analysis of variance followed by Bonferroni's test was used to test the significance of differences. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Response of Ppv to antigen

After injection of L-NAME or D-NAME into the blood-free constant-flow perfused liver, Ppv did not change significantly. As shown in Fig. 1, after injection of 0.1 mg ovalbumin into a D-NAME-pretreated liver, venoconstriction occurred, as evidenced by an increase in Ppv. The Ppv reached peak levels of 21.4 ± 1.1 cmH₂O 3 min after the injection of antigen from a baseline value of 7.7 ± 0.1 cmH₂O. The Ppv-to-Phv gradient, the determinant of Rt, increased 2.9-fold baseline, indicating that Rt increased by the same degree, as indicated in Table 1. In fact, Rt increased from a baseline value of 0.17 ± 0.01 to 0.47 ± 0.04 cmH₂O/mL per min per 10 g. Thereafter, Ppv and Rt decreased gradually towards baseline values.

In the L-NAME group, Ppv also increased after the injection of antigen, but the peak Ppv levels of 27.4 ± 0.8 cmH₂O 3 min after antigen injection were significantly greater than those seen in the D-NAME group (21.4 ± 1.1 cmH₂O). Thus, the peak Rt level in the L-NAME group was 1.7-fold greater than that in the D-NAME group. This significant difference was observed until 10 min after antigen injection (Fig. 1). However, as shown in Fig. 1, the augmentation of antigen-induced venoconstriction induced by L-NAME was

Table 1 Effects of N^G -nitro-L-arginine methyl ester (L-NAME) and N^G -nitro-D-arginine methyl ester (D-NAME) on haemodynamic variables and NO_x after antigen injection in rat isolated perfused livers

Groups	L-NAME			D-NAME			L-Arginine + L-NAME		
	Pretreatment	Baseline	3 min	Pretreatment	Baseline	3 min	Pretreatment	Baseline	3 min
Ppv (cmH ₂ O)	7.2 ± 0.2	7.8 ± 0.4	27.4 ± 0.8*	7.4 ± 0.1	7.7 ± 0.1	21.4 ± 1.1*	7.4 ± 0.2	8.0 ± 0.4	22.6 ± 1.4*
Phv (cmH ₂ O)	0.3 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Qpv (mL/min per 10 g)	41 ± 3	41 ± 3	41 ± 3	42 ± 2	42 ± 2	42 ± 2	38 ± 1	38 ± 1	38 ± 1
Rt	0.19 ± 0.03	0.20 ± 0.04	0.74 ± 0.10*	0.17 ± 0.01	0.17 ± 0.01	0.47 ± 0.04*	0.19 ± 0.01	0.20 ± 0.01	0.59 ± 0.04*
NO_x concentration (μmol/L)									
Inflow	0.45 ± 0.05	0.51 ± 0.05	0.54 ± 0.05	0.45 ± 0.02	0.52 ± 0.02	0.55 ± 0.02	0.40 ± 0.05	0.50 ± 0.05	0.52 ± 0.05
Outflow	0.45 ± 0.06	0.50 ± 0.06	0.54 ± 0.06	0.53 ± 0.03	0.45 ± 0.06	0.54 ± 0.02	0.41 ± 0.05	0.48 ± 0.05	0.51 ± 0.06

Values are the mean ± SEM. * $P < 0.05$ compared with baseline.

Ppv, portal vein pressure; Phv, hepatic venous pressure; Qpv, perfusate flow; Rt, vascular resistance (in cmH₂O/mL per min per 10 g).

reversed by additional perfusion with L-arginine (1 mmol/L). The pressure response in the L-arginine + L-NAME group was similar in magnitude to that in the D-NAME group. These results suggest that the anaphylactic reaction in rat livers produced NO, which then attenuated the anaphylactic hepatic venoconstriction.

Perfusate concentrations of NO_x

Perfusate levels of either NO_2^- or NO_3^- did not change significantly after antigen injection in any group studied (Table 1). Moreover, differences in NO_2^- and NO_3^- between the outflow and inflow did not change significantly in any group and the differences between the two groups were not significant throughout the experimental period, as shown in Fig. 2.

DISCUSSION

The present study showed that the NOS inhibitor L-NAME augmented anaphylactic venoconstriction in rat isolated livers perfused with blood-free solution under constant flow. L-Arginine completely reversed enhancement of the anaphylactic venoconstriction by L-NAME. This finding suggests that, similar to guinea-pig livers,⁵ rat livers produce NO during the anaphylactic reaction and that the NO released then attenuates anaphylactic hepatic venoconstriction. We also measured perfusate concentrations of NO metabolites before and after antigen in order to confirm the production of NO. However, unexpectedly, we could not detect significant differences in perfusate NO_x concentrations between the outflow and inflow perfusate, even though we used the highly sensitive HPLC–Griess system (ENO-10) for measurement of NO_x .

We have reported previously that anaphylactic venoconstriction occurs in rat isolated blood-perfused livers.⁷ In the present study, a similar response was observed in rat livers perfused with blood-free solution. This indicates that the intrahepatic mast cells rather than circulating blood cells, such as basophils and platelets, were involved in the hepatic regional anaphylaxis in the present study.

N^G -Nitro-L-arginine methyl ester is widely used to inhibit endothelial synthesis of NO. However, multiple non-specific actions of L-NAME other than simple inhibition of NO synthesis have also been reported.^{15–18} These include generation of superoxide anions and prostaglandin endoperoxides, both of which cause vaso-

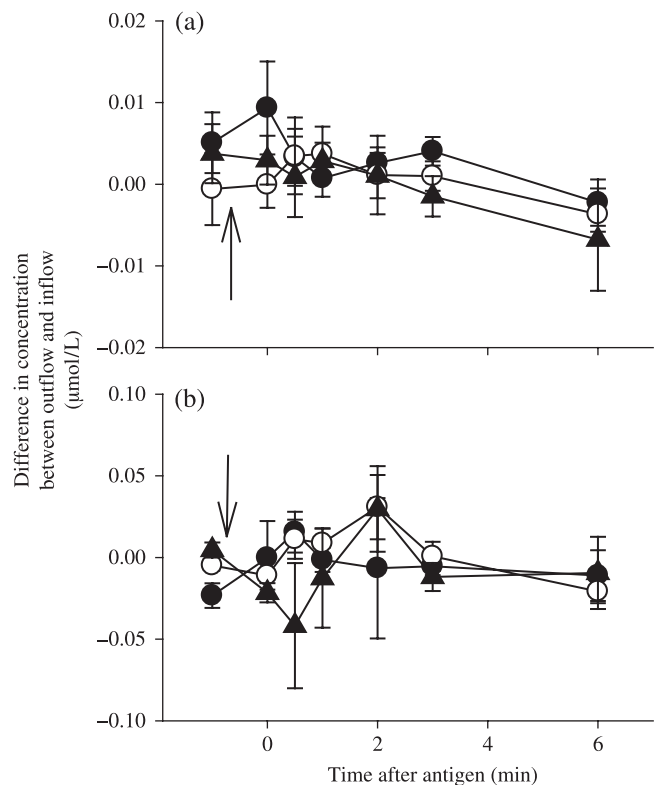


Fig. 2 Summary of changes in differences in (a) NO_2^- and (b) NO_3^- concentrations between inflow and outflow perfusate after antigen injection in the N^G -nitro-D-arginine methyl ester (D-NAME; ○), N^G -nitro-L-arginine methyl ester (L-NAME; ●) and L-arginine + L-NAME (▲) groups. The arrows indicate the time of pretreatment. Data are the mean ± SEM ($n = 5$). * $P < 0.05$ compared with baseline.

constriction. Thus, NO-independent mechanism may have been involved in the effect of L-NAME in the present study. However, we found that the addition of L-arginine completely reversed the augmentation by L-NAME. This finding suggests that the effect of L-NAME is due to inhibition of NO synthesis rather than due to the production of vasoconstrictors, such as superoxide anions and prostaglandin endoperoxide.

The mechanism responsible for NO release during hepatic anaphylaxis may be ascribed to shear stress-dependent and -independent mechanisms.¹⁹ In rat livers perfused at constant flow, anaphylaxis contracted hepatic vessels, where shear stress could be increased, resulting in NO release from the constricted endothelium. Conversely, the shear stress-independent mechanism may be related to humoral substances released by the anaphylactic reaction *per se*. We assume that chemical mediators, such as platelet-activating factor (PAF), thromboxane (TX) A₂, histamine, cysteinyl leukotrienes and serotonin, may act on their corresponding receptors on the endothelium, resulting in the subsequent activation of endothelial (e) NOS and production of NO. In fact, it has been reported that most mediators of anaphylaxis, such as histamine,^{20,21} leukotrienes,²² TXA₂²¹ and PAF,²³ all stimulate NO release from the vascular endothelium.

The percentage proportion of NO counter-regulation in anaphylactic venoconstriction of the portal vein in rats was approximately 28% (6.0 cmH₂O). This seems to be smaller than that of similarly examined guinea-pig livers, in which NO inhibition augmented the anaphylaxis-induced increase in Ppv by 80% (18.1 cmH₂O).⁹ The relatively lesser augmentation induced by L-NAME in the present study may be ascribed to differences in the perfusate, as well as species differences. The Krebs' solution used in the present study had a very low viscosity and, thus, produced a low vascular resistance, resulting in low shear stress and small NO production compared with the diluted blood used in the previous guinea-pig liver study.⁶ The reason why we used blood-free Krebs' solution as the perfusate in the present study was that a solution including free haemoglobin could prevent accurate measurement of perfusate NO₂⁻ and NO₃⁻ concentrations.²⁴ Haemolysis and, thus, the presence of free haemoglobin in the perfusate could not be avoided in the present experimental set up including a roller pump. Because NO₂⁻ has been suggested to be a more sensitive and specific marker of NO than NO₃⁻²⁵ and it has been suggested that NO₂⁻ is the main metabolite of NO in haemoglobin-free medium,²⁶ we endeavoured to quantify the substance at nmol/L levels. The possible reasons why the highly sensitive HPLC–Griess system could not detect the expected increase in NO₂⁻ as a reflection of enhanced NO release during anaphylaxis in rat isolated perfused livers remain obscure. However, some possible causes may be derived from the following. The device we used may still be insufficient to detect smaller changes in NO₂⁻ from the vascular bed in an excised organ with a large volume of perfusate compared with changes in a whole body and the substance may be easily influenced by the surrounding atmosphere. In addition, we have experienced disappearance of NO₂⁻ or NO₃⁻ after perfusion of certain organs.^{12,13}

In conclusion, we are able to present functional evidence that NO production may be increased during rat hepatic anaphylaxis. This hepatic anaphylactic response was independent of circulating blood cells. However, perfusate NO_x measurements using the highly sensitive HPLC–Griess system could not detect physical evidence of increased intrahepatic NO production in rat isolated perfused anaphylactic livers; thus, this issue requires further examination.

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