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Therapeutic Effects of Various Nitric Oxide Synthase Inhibitors on Septic Shock: Influence on Hemodynamics and Histopathological Changes

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The effects of nitric oxide synthase (NOS) inhibitors were investigated on the hemodynamics, nitric oxide (NO) production, coagulofibrinolytic system and histopathological changes to prove their therapeutic efficacy in septic shock. Their hypertensive effects were compared with those of dobutamine (DOB). Rats under pentobarbital anesthesia were treated with endotoxin (5 mg/kg i.v.) to produce endotoxin shock. The NOS inhibitors used were: N^G-monomethyl-L-arginine (L-NMMA, a non-selective NOS inhibitor), S-methylisothiourea (SMT, a relatively selective inducible NOS inhibitor or iNOS), and ONO-1714 (a more selective iNOS inhibitor). Following an early quiescent state, NO production began gradually 1 to 2 hr after endotoxin administration and peaked in 4 to 6 hr resulting in hypotensive shock. Administering NOS inhibitors or DOB during the shock stage produced a marked amelioration of hypotension with SMT or ONO-1714, a slight improvement with L-NMMA, and no significant change with DOB. After endotoxin administration, the kidney, lung, liver, and small intestine, examined for histopathological changes, showed hemorrhage, edema, necrosis, and intravascular microthrombosis. The NOS inhibitors failed to correct these conditions, even exacerbating in some (a mechanism for the actions involved was discussed). The tissue damage and its treatment should be investigated further when NOS inhibitors are used to treat septic shock.

Key words: therapeutic effects in septic shock, hemodynamics, NO production, histopathology, NOS inhibitors

Introduction

In septic shock, a large quantity of nitric oxide (NO) is produced by the action of inducible NO synthase (iNOS), which in turn is induced by endotoxin and pro-inflammatory cytokines; NO then acts on the blood vessels to cause a marked hypotension and damages the myocardium and other tissues[1)～5)]. NOS inhibitors have been investigated as therapeutic agents for such conditions[6)～8)]. Administration of non-selective NOS inhibitors, because of their inhibition of constitutive NOS (cNOS), provokes tissue damage or organ failure through a marked hypoperfusion of tissues and organs[9)～11)]. Inhibition of iNOS suppresses excessive production of NO by iNOS that is induced by bacterial invasion, and reduces hypotension and tissue damage[12)～14)]. In animal experiments, it has been reported that these iNOS inhibitors reduce mortality[12)～14)]. Thus, their

clinical application may be expected.

For septic shock, the therapeutic value of various adrenergic agents is limited; so dopamine or dobutamine (DOB) has been used for treatment[15]. It has been reported that DOB is effective in inhibiting the release of NO[16].

In the present study, the therapeutic effects of NOS inhibitors on the hemodynamics and histopathological changes in cases of endotoxin shock were investigated. These effects were also compared with the hemodynamic effects of DOB.

Methods

The handling and treatment of the experimental animals for this study were approved by the Institutional Committee of Tokyo Women's Medical University.

Male Sprague-Dawley rats weighing 350 to 450 g were used under pentobarbital anesthesia (50 mg/kg ip). The carotid and femoral arteries, which had been catheterized by a polyethylene tube (PE50), were used to obtain blood samples and monitor arterial pressure. The rats underwent a laparotomy after catheterization. An NO-selective thin electrode (200 μm, Intermedical Co., Nagoya, Japan) was inserted orthoptically into the subcapsular kidney tissue and retained there. NO production was recorded in real time via the NO electrode, which was connected to an NO meter (Model 501, Intermedical Co.)[17]. About 30 min later—after the NO electrode had been inserted and the blood pressure and NO level had stabilized—endotoxin (5 mg/kg iv, LPS from *E. coli*; DIFCO Laboratories, Detroit, MI, USA) was administered intravenously to induce a septic shock[18].

The blood pressure began to decline slowly 60 to 90 min following endotoxin administration, resulting in extreme hypotension within 3 to 4 hr (Fig. 1). The NOS inhibitors and DOB (5 mg/kg) were given 2 to 3 hr after endotoxin administration and the subsequent changes were observed. Then about 2 hr later a blood sample was obtained and tissue specimens were collected for a histopathological examination. The NOS inhibitors used were a nonselective NOS inhibitor (having an almost equal affinity for cNOS and iNOS) N^G-monomethyl-L-arginine (L-NMMA 50 mg/kg, Calbiochem-Novabiochem Co., USA), a relatively selective iNOS inhibitor, S-methylisothiourea (SMT 5 mg/kg, Aldrich Chemical Co., USA), and a more selective iNOS inhibitor (1S, 5S, 6R, 7R)-7-chloro-3-imino-5-methyl-2-azabicyclo-[4,1,0] heptane hydrochloride (ONO-1714 0.1 mg/kg, ONO Pharmaceutical Co., Osaka, Japan)[19].

After endotoxin administration, L-NMMA, SMT, ONO-1714 or saline (control) was given (each group, $n = 6$) and their effects on NO production, blood pressure, contents of coagulofibrinolytic factors and histopathological changes were observed. For the investigation of coagulofibrinolytic factors, blood samples were drawn to determine the following: platelet count (via an electrical resistance method), serum fibrin and fibrinogen degradation products (FDP) analysis using FDP-E (MBL Co., Nagoya, Japan), plasma fibrinogen analysis (cyan-methemoglobin method), and antithrombin III (AT-III) determination using AT-III spectrophotometry with a coupler, SMT III (Daiichi Kagaku-Yakuhin Co., Tokyo, Japan).

After the experiment, the kidney, lung, liver and intestine were excised, fixed in a 10% formalin solution and preserved for later investigation. 4 to 6 μm-thick tissue sections, after staining with hematoxylin-eosin (HE) and phosphotungustic acid-hematoxylin (PTAH) for fibrin, were examined for possible histopathological changes.

Statistical analysis was performed as follows. The results were indicated as mean ± SEM (standard error of the mean). Student's paired and unpaired t-tests were used to compare the means within and between groups, respectively, the level of significance being set at $p < 0.05$.

Results

1. NO production in tissue and blood pressure

Changes in NO production in the tissue following endotoxin administration were recorded in real time with an NO-selective electrode set in the kidney tissue. Following endotoxin administration, NO production was almost undetectable during the early stage. However, the NO level began to increase slowly after 60 to 90 min, and rapidly after 4 hr, peaking 6 hr after administration. With the pro-

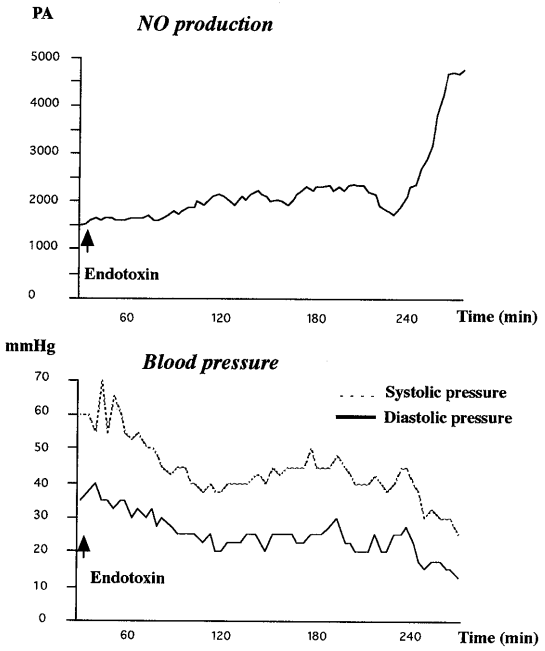


Fig. 1 Changes of NO production and blood pressure following endotoxin administration
NO: nitric oxide

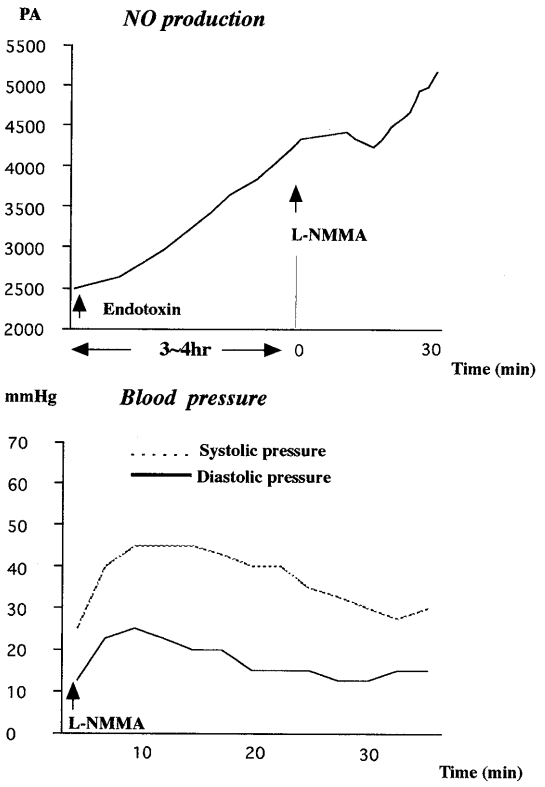


Fig. 2 Changes of NO production and blood pressure at the time of L-NMMA application following endotoxin administration
NO: nitric oxid, L-NMMA: N^G-monomethyl-L-arginine.

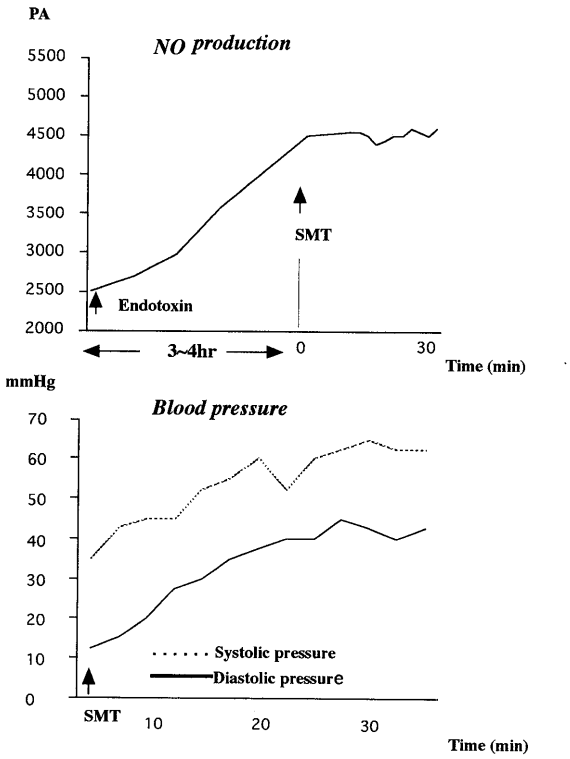


Fig. 3 Changes of NO production and blood pressure at the time of SMT application following endotoxin administration
NO: nitric oxide, SMT: S-methylisothiourea.

gress in NO production, blood pressure decreased gradually and reached the 20 to 30 mmHg level 4 to 6 hr after endotoxin administration (Fig. 1).

When NO production increased considerably about 3 hr after endotoxin administration, the application of L-NMMA (50 mg/kg iv) inhibited the reaction slightly, with an associated transient rise in blood pressure (Fig. 2). The hypotensive state was normalized markedly by the application of SMT (5 mg/kg iv, Fig. 3). The effect of ONO-1714 was investigated at 3 different dosage levels: 0.01, 0.05 and 0.1 mg/kg iv. With the first two dosages, inhibition of NO production and recovery from hypotension were only slight, but at 0.1 mg/kg, NO production was clearly inhibited, and the hypotensive state was normalized more markedly than at SMT administration (Fig. 4). The effect of NOS inhibitors on the blood pressure following endotoxin administration is shown in Table 1. Neither the application of DOB (5 mg/kg iv) 3 hr after endotoxin administration (Fig. 5) nor the saline application (a control) in-

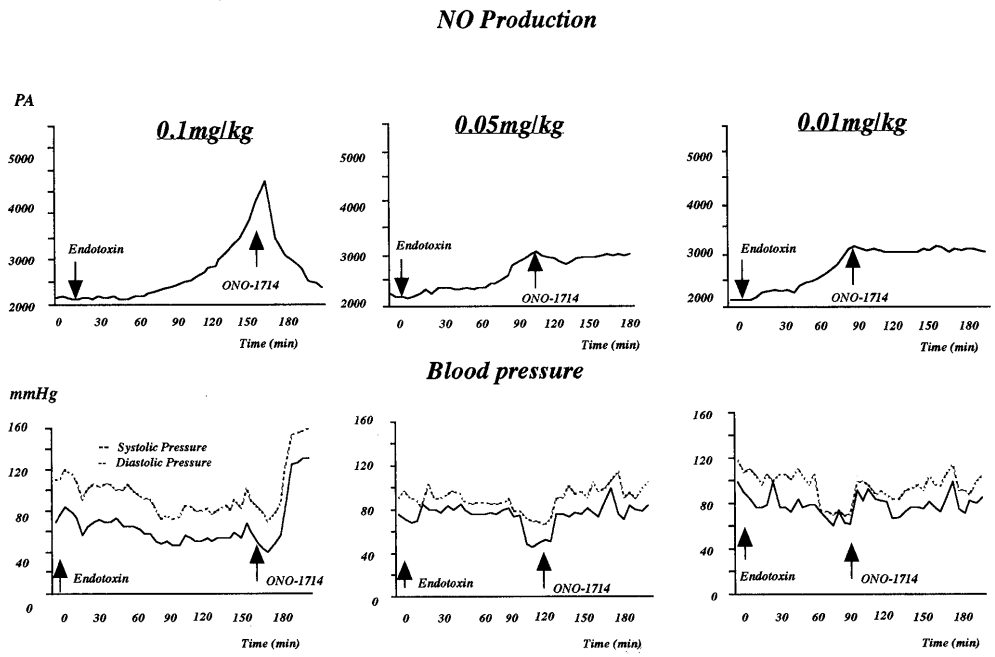


Fig. 4 Changes of NO production and blood pressure at the time of ONO-1714 application following endotoxin administration
NO: nitric oxide.

Table 1 Effect of NOS inhibitors on the blood pressure following endotoxin administration

	Rate of amelioration (%)			
	10 min after treatment		60 min after treatment	
	Systolic pressure	Diastolic pressure	Systolic pressure	Diastolic pressure
Endotoxin (n = 9)	36.2 ± 6.87	24.0 ± 4.89	3.4 ± 5.17	9.4 ± 4.98
	*	**	ns	ns
Endotoxin + L-NMMA (n = 7)	54.8 ± 6.02	60.6 ± 7.09	10.8 ± 9.93	18.6 ± 7.09
	**	**	**	**
Endotoxin + SMT (n = 9)	83.2 ± 13.8	81.0 ± 4.18	105.0 ± 16.75	160.4 ± 6.80
	**	*	ns	ns
Endotoxin + DOB (n = 7)	12.0 ± 4.32	6.0 ± 6.93	16.2 ± 13.45	15.0 ± 18.82

Values are expressed as the mean ± SEM. n: number of experimental animals, ns: not significant, *: $p < 0.05$, **: $p < 0.01$, L-NMMA: N^G-monomethyl-L-arginine, SMT: S-methylisothiourea, DOB: dobutamine.

duced significant changes in NO production or blood pressure.

2. Coagulofibrinolytic system and histopathological changes

The effects of treatment with NOS inhibitors on changes in the platelet count and the content of coagulofibrinolytic factors following endotoxin administration are shown in Table 2. The decrease in platelet count caused by endotoxin administration was normalized significantly by the SMT treatment ($p < 0.05$), but the corrective effect of L-NMMA was not significant. The FDP level increased with endotoxin administration, and decreased significantly with SMT treatment ($p < 0.01$) and with L-NMMA ($p < 0.05$). The soluble fibrin (Fibr) level that had been reduced by endotoxin administration increased significantly by the treatment with SMT, but did not change significantly with L-NMMA. The AT-III level that had been reduced by endotoxin administration did not change significantly by

the treatment with SMT or L-NMMA.

The histopathological findings for the endotoxin shock are shown in Fig. 6. The presence of thrombi, focal necrosis, and hemorrhage were noted in the kidney and lung. Hemorrhage combined with local necrosis were noted in the liver, and subserosal and submucosal hemorrhages were noted in the intestine. These findings did not ameliorate significantly despite treatment with NOS inhibitors; instead they appeared to be slightly aggravated. The histopathological findings were almost uniform after being treated with L-NMMA, SMT and ONO-1714 (Fig. 7). DOB did not induce a significant change in histopathological findings following endotoxin administration.

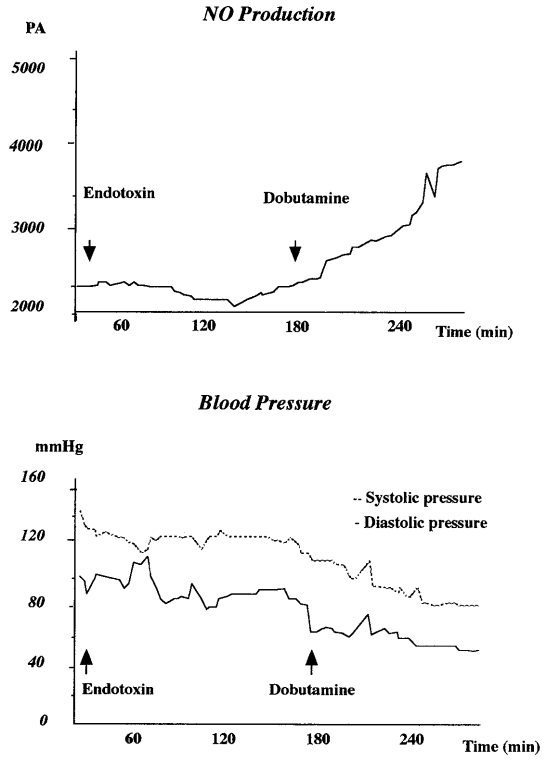


Fig. 5 Changes of NO production and blood pressure at the time of dobutamine application following endotoxin administration
NO: nitric oxide.

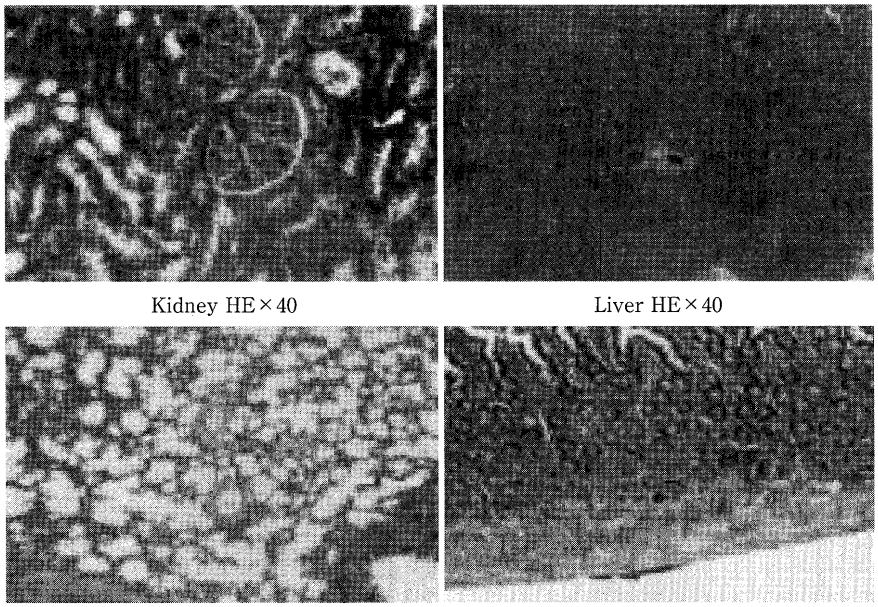
Discussion

Hypotension and depression of myocardial contractility, which may be attributable to NO action, are the characteristic features of septic shock[4)5)]. In treating septic shock, the therapeutic value of various adrenergic agents is limited, and so therapy with drugs such as dopamine and DOB is recommended[20)]. DOB has been reported to augment myocardial contractility without noticeably affecting the heart rate or peripheral resistance in clinical patients[20)]. It has also been reported that this agent has an inhibitory effect on NO release[16)]. These mechanisms of action were the reason that DOB was included in the present study. However, DOB failed to affect NO production, which was caused by endotoxin administration, and it did not affect the blood pressure to any significant extent. DOB had been reported to improve myocardial contraction, blood flow, and in particular, the O_2 supply to the

Table 2 Effect NOS inhibitors on the platelet and coagulofibrinolytic system following endotoxin administartion

	Coagulofibrinolytic system			
	Plt ($\times 10^3/\mu l$)	FDP ($\mu g/ml$)	Fibr (mg/dl)	AT-III (mg/dl)
Control (n = 7)	102.43 ± 6.07	< 5	133.57 ± 7.79	108.28 ± 1.98
Endotoxin (n = 4)	12.32 ± 1.21	> 40	20.00	59.00 ± 2.58
	*	**	**	ns
Endotoxin + SMT (n = 4)	60.60 ± 4.77	< 5	80.75 ± 3.94	42.00 ± 7.11
	ns	*	ns	ns
Endotoxin + L-NMMA (n = 4)	23.25 ± 4.29	10 << 40	20.00	63.00 ± 11.00

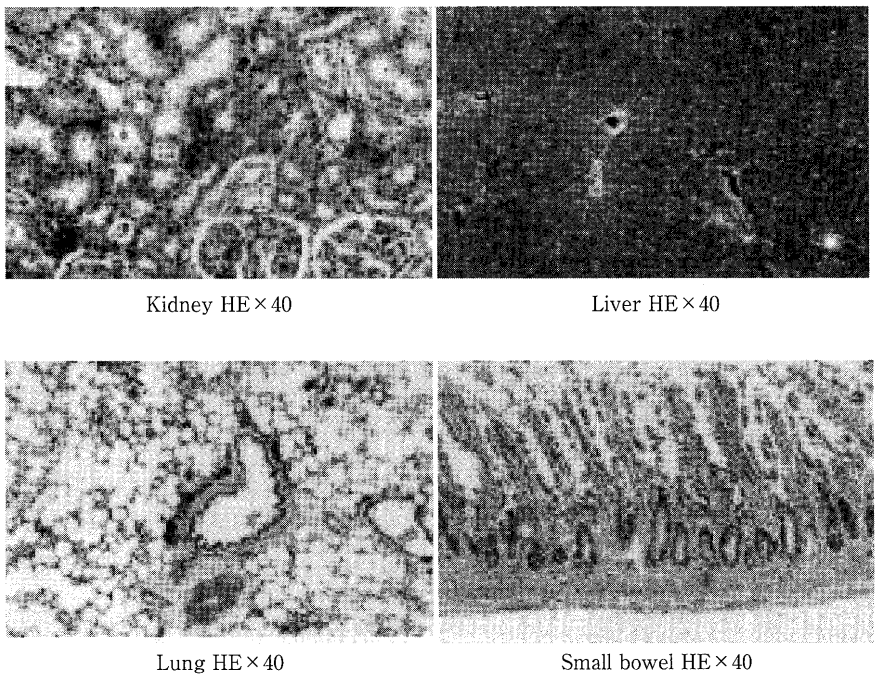
Values are expressed as the mean ± SEM. n: number of experimental animals, ns: not significant, *: $p < 0.05$, **: $p < 0.01$, Plt: platlet, FDP: fibrin degradation products, Fibr: soluble fibrin, AT-III: antithrombin, SMT: S-methylisothiourea, L-NMMA: N^G-monomethyl-L-arginine.



Kidney HE×40　　Liver HE×40

Lung HE×40　　Small bowel HE×40

Fig. 6 Histopathological changes produced by endotoxin administration
The presence of thrombi, focal necrosis and hemorrhage in the kidney and lung, and hemorrhage combined with local necrosis in the liver were noted.



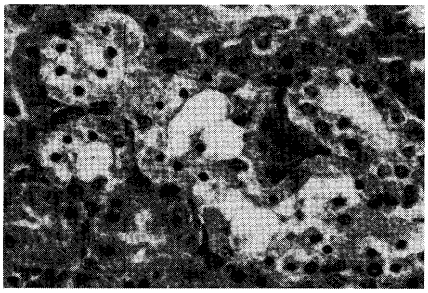
Kidney HE×40　　Liver HE×40

Lung HE×40　　Small bowel HE×40

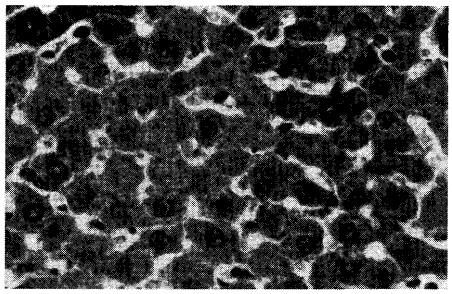
Fig. 7 Histopathological changes produced by ONO-1714, an NOS inhibitor, application following endotoxine administration
The findings did not ameliorate significantly with NOS inhibitors, and they appeared to be slightly aggravated. The histopatholgical findings were almost uniform after treatment with L-NMMA, SMT, and ONO-1714.

tissues during shock[21]; the results given here failed to attest to these effects.

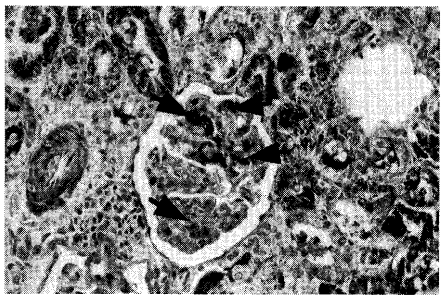
In septic shock, proinflammatory cytokines and fissue facter are released from macrophages, endothelial cells, and neutrophils, the condition results in a marked increase in thrombin and fibrin produc-



Kidney HE×100



Liver HE×100



Kidney PTAH×80

Fig. 8 High magnification of photomicrographs of liver and kidney tissue and PTAH staining of kidney fissue
A moderate neutrophil infiltration and microthrombosis could be seen.

tion, ultimately culminating in the syndrome of disseminated intravascular coagulation (DIC)[22)~25)]. We have previously confirmed the development of DIC with endotoxin administration in rats that were placed under conditions identical to those set forth in this experiment[26)].

In the current experiment with endotoxin shock, iNOS inhibitors inhibited NO production; consequently hypotension ameliorated markedly, while histopathological changes remained unaffected or even exacerbated to some degree. Thus, to explain these findings, the following was suggested; although iNOS inhibitors mitigate hypotension, histopathological changes produced as the inflammatory response of tissue provoked by END[27)] is not inhibited by these inhibitor. As NO has cytoprotective roles, such as inactivation of oxygen free radicals, prevention of cardiovascular thrombosis, inhibition of platelet aggregation and leukocyte adhesion[28)~30)]. The loss of NO caused by NOS inhibitors results in promoting thrombosis formation, platelet aggregation and tissue damage. Although a reduction in hypotension alleviates DIC of the endotoxic effect, a loss of NO aggravates tissue damage, as noted above. Thus, the histopathological changes observed in the present study, indicates an integrative inflammatory effects of END.

Jourdain et al[31)] reported that, after having confirmed the development of DIC when pigs were subjected to septic shock (induced by endotoxin), the administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor, caused aggravated changes related to DIC in a histopathological presentation and in the coagulation system. Because L-NAME is a non-selective NOS inhibitor, it cannot be denied that severe vasoconstriction of the blood vessels supplying the organs was caused by cNOS inhibition, which is responsible for these histopathological changes. They did state in their discussion that with the presence of more advanced microthrombosis, histologic changes in the kidney and lung were worse in L-NAME-treated animals. Radomsky[32)] also suggested that NOS inhibitors may lead to an increase in thrombogenicity of the endothelium and the platelet thrombin content in the vasculature[31)]. Thus, he proposed that there was a microthrombus-promoting effect of NOS inhibitors in addition to severe vasoconstriction of blood

vessels supplying various organs. With regard to the use of NOS inhibitors as therapeutic agents for septic shock, the selectivity of the iNOS inhibitor has been the focal point up to now. However, because the development of DIC in severe disorders like septic shock has been cited frequently, the mechanism by which tissue damage is provoked with the use of these inhibitors, as well as the remedy for such damage, should also be investigated further.

Conclusions

The therapeutic effects of NOS inhibitors for endotoxin shock in relation to hemodynamics and histopathological changes were investigated in rats. Although iNOS inhibitors showed a remarkably ameliorative effect on hypotension, their effects on histopathological changes were practically nonexistent (or even aggravating in some instances). Thus, the application of NOS inhibitors for endotoxin shock should be investigated further, vis-á-vis tissue damage and its possible countermeasures, in addition to the selectivity of iNOS inhibitors.

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敗血症性ショックに対する各種の一酸化窒素合成酵素阻害剤の治療効果
—血行動態および組織病理学的変化に対する影響—

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敗血症性ショックに対する一酸化窒素合成酵素（NOS）阻害剤の治療効果を，血行動態，組織における一酸化窒素（NO）産生，凝固線溶系および組織病理学的変化に対する影響について検討した．エンドトキシンショックに対するNOS阻害剤の昇圧効果はドブタミン（DOB）の昇圧効果と比較検討した．ペントバルビタール麻酔ラットを用い，エンドトキシン（5mg/kg iv）適用によりショックを発生させた．NOS阻害剤としては，非選択的NOS阻害剤 N^{G}-monomethyl-L-arginine（L-NMMA），相対的誘導型NOS（iNOS）阻害剤S-methylisothiourea（SMT）およびiNOSに親和性のより高いONO-1714を用いた．エンドトキシンの静脈内適用により，初期にはNO産生は認められず，1～2時間後より徐々にNO産生がみられるようになり，4～6時間後にピークとなり，血圧はショック状態となった．この時NOS阻害剤あるいはDOBを投与すると，SMTおよびONO-1714では顕著な低血圧の改善がみられたが，L-NMMAの作用は軽度であり，DOBでは有意な血圧の変化はみられなかった．エンドトキシン適用により，組織病理学的変化として腎，肺，肝および小腸について検討したが，組織の出血，浮腫，壊死および血管内微小血栓が認められ，これらはNOS阻害剤の投与により，改善はみられず，却って一部組織像の増悪がみられた．エンドトキシンショック時のNOの増加による低血圧はNOS阻害剤によるNOの除去により改善されるが，侵襲時のサイトカイン放出による組織因子の遊離および白血球の活性化を介する組織傷害は，NOS-NO系と関係なく，NOS阻害剤の影響を受けず，却ってNOの除去は組織因子等の作用を増強して，組織像の一部増悪がみられたと考える．敗血症性ショックに対するNOS阻害剤による治療に際して，この組織傷害性への対処の検討が必要であると考える．