ELSEVIER

Biochemical Pharmacology 63 (2002) 853–858

Biochemical Pharmacology

Effects of sanguiin H-6, a component of Sanguisorbae Radix, on lipopolysaccharide-stimulated nitric oxide production

Takako Yokozawa[a,*], Cui Ping Chen[a], Takashi Tanaka[b], Kenichi Kitani[c]

[a]*Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*
[b]*Faculty of Pharmaceutical Sciences, Nagasaki University, Nagasaki 852-8131, Japan*
[c]*National Institute for Longevity Sciences, Obu 474-0031, Japan*

Received 19 March 2001; accepted 27 July 2001

Abstract

The present study was conducted to evaluate the effect of sanguiin H-6, a component of Sanguisorbae Radix, on the production of nitric oxide (NO), using macrophages activated by lipopolysaccharide (LPS). Sanguiin H-6 inhibited nitrite production, taken as an index for NO, in a concentration-dependent fashion. This compound decreased inducible NO synthase (iNOS) activity, with the inhibitory effect at a concentration of 25 μM being equal to that of the known iNOS inhibitor aminoguanidine at 50 μM. However, unlike aminoguanidine, sanguiin H-6 was associated with improved cell viability. Reverse transcription-polymerase chain reaction analysis revealed that the expression of iNOS mRNA in activated macrophages was suppressed by sanguiin H-6 in a concentration-dependent manner. In addition, sanguiin H-6 even at a low concentration showed a clear scavenging effect on the NO generated from sodium nitroprusside (an NO donor). These findings indicate that not only does sanguiin H-6 act directly as an NO scavenger, but it also inhibits NO production in LPS-activated macrophages by the concomitant inhibition of iNOS mRNA induction and enzyme activity.

Keywords: Sanguiin H-6; Sanguisorbae Radix; Nitric oxide; Inducible nitric oxide synthase; Macrophages

1. Introduction

During infection, inflammation, or an immune response, macrophages *in vivo* are stimulated to rapidly metabolize L-arginine (L-Arg) in the presence of nitric oxide synthase (NOS), and NO is produced as a result of this reaction. NO, together with active oxygen and other antimicrobial substances and antitumor agents produced by macrophages, plays an important role in protecting the body by causing damage to tumor cells and preventing infection with microorganisms. On the other hand, excessive production of NO can cause a state of shock, damage to tissues of the living body, and unfavorable conditions, such as autoimmune disease [1–3]. Thus, NO is deeply involved in the immune system and defense mechanisms of the body, and because of this, much attention has been focused on the importance of its actions.

NO is a radical gas synthesized by NOS. It has three isoforms, and the NO produced varies widely in its physiological significance according to the isoform of NOS involved in the synthesis. The physiological role of NO is enhanced when it is synthesized by neuronal NOS or endothelial NOS, whereas the pathological role is enhanced when it is synthesized by iNOS [4,5]. It has been speculated that, once induced, iNOS synthesizes a large amount of NO, and thus, causes a marked increase in the local concentration of NO, allowing the predominance of NO as a radical [1]. The NO radical is also likely to be produced in the environment where O_2^- coexists, and it can be converted to a more reactive radical through a reaction with O_2^- [6]. Considering these characteristic features of NO as a radical, together with the fact that iNOS is readily induced in mouse and rat inflammatory cells after treatment with LPS or inflammatory cytokines [7,8], iNOS seems to be deeply involved in the processes of the immune reaction and inflammation. From this viewpoint, attention has been paid to the role of iNOS inhibitors, and it has been reported

* Corresponding author. Tel.: +81-76-434-7631; fax: +81-76-434-4656.
E-mail address: yokozawa@ms.toyama-mpu.ac.jp (T. Yokozawa).

Abbreviations: DTT, dithiothreitol; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; iNOS, inducible nitric oxide synthase; L-Arg, L-arginine; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; NO, nitric oxide; NOS, nitric oxide synthase; O_2^-, superoxide; RT-PCR, reverse transcription-polymerase chain reaction.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00930-3

that L-canavanine, N^{ω}-amino-L-arginine, N^{ω}-iminoethyl-L-ornithine, *S*-methylisothiourea, and aminoguanidine can potently inhibit macrophage-derived iNOS [5,9,10].

We searched for iNOS inhibitors among Oriental medicines, and carried out a study to screen for their effects on excessive NO in an *in vitro* evaluation system using sodium nitroprusside, an NO donor. As a result, we found a new beneficial effect of Sanguisorbae Radix, reflected in the finding that Sanguisorbae Radix extract markedly inhibits excessive NO [11,12]. In a subsequent study, we investigated which component was responsible for the inhibitory effect of the extract, and found high activity in sanguiin H-6 [13]. Sanguisorbae Radix is an Oriental medicine not commonly used in Japan, and not present in any Oriental medical prescriptions currently available in the clinical setting. However, in China, this drug is taken internally for hemostasis or treatment of hematemesis, hemoptysis, melena, and hypermenorrhea, and externally for the treatment of dermatitis, eczema, and incised wounds. Previous pharmacological studies of this drug have been restricted to its effects on burn injuries and hemostatic or antiemetic effects in rabbits and pigeons, and its antimicrobial effects on *Staphylococcus aureus* and *Pseudomonas aeruginosa* [14]. In this regard, much is expected from the effects of sanguiin H-6 isolated from Sanguisorbae Radix.

In the present study, we investigated the mechanism by which sanguiin H-6 acts, with special reference to iNOS.

2. Materials and methods

2.1. Sanguiin H-6

As described previously [13], roots of Sanguisorbae Radix (*Sanguisorba officinalis* L., Rosaceae), grown in China and supplied by the Uchida Wakan-yaku Co. Ltd., were ground to a fine powder before being extracted with acetone and water (7:3, v/v). The extract was then partitioned three times with ethyl ether, and the water-insoluble precipitate in the aqueous layer was collected by filtration. The filtrate was applied to a column of Diaion HP-20SS and eluted with water containing increasing proportions of methanol. Elution of the column with 0–60% methanol gave the tannin-containing fraction. This fraction was separated by Sephadex LH-20 column chromatography with 0–100% methanol and then with water–acetone. A part of the fractions was separated further by Sephadex LH-20 column chromatography with ethanol containing increasing proportions of a water and acetone mixture (1:1, v/v) to yield sanguiin H-6. The level of sanguiin H-6 in the aqueous extract of Sanguisorbae Radix was estimated to be about 0.7%. The chemical structure is shown in Fig. 1.

2.2. Medium and reagents

RPMI 1640 medium and fetal bovine serum (FBS) were purchased from the Nissui Pharmaceutical Co. Ltd. and Cell Culture Laboratories, respectively. Thioglycollate broth and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were obtained from Wako Pure Chemical Industries, Ltd. LPS, pepstatin A, chymostatin, aprotinin, phenylmethylsulfonyl fluoride, FAD, tetrahydrobiopterin, dithiothreitol (DTT), NADPH, lactate dehydrogenase (LDH), sodium pyruvate, and aminoguanidine were all from the Sigma Chemical Co. Deoxyribonuclease I, dNTP mix, RNase inhibitor, RNase H-reverse transcriptase, ribonuclease A, and Taq DNA polymerase were from Takara Shuzo. Other reagents were of the highest grade available.

2.3. Animals

Male BALB/c strain mice (Shizuoka Agricultural Cooperative Association for Laboratory Animals) were used at 6

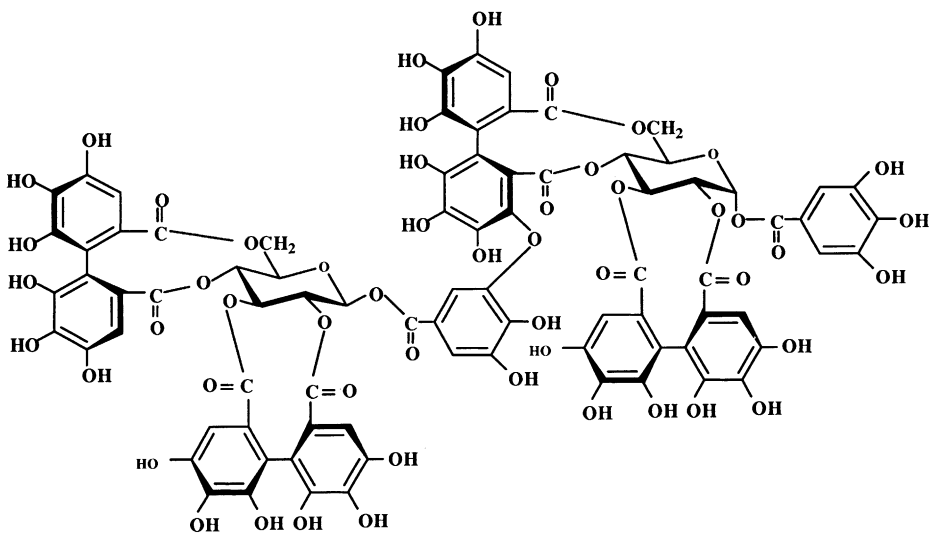

Fig. 1. Structural formula of sanguiin H-6.

weeks of age. They were kept in an air-conditioned room with lighting from 7:00 a.m. to 7:00 p.m. and given commercial pellet chow (Clea Japan Inc.; comprising 24% protein, 3.5% lipid, and 60.5% carbohydrate) and water *ad libitum*. The experiments were conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals at Toyama Medical and Pharmaceutical University.

2.4. Cell culture

According to the method of Lefkowitz *et al.* [15], mice were injected intraperitoneally with 1.5 mL of 4% thioglycollate broth. After 4 days the animals were killed by cervical dislocation. Peritoneal lavage was performed using cold RPMI 1640 medium. Peritoneal macrophages were centrifuged at 1000 *g* for 2 min at room temperature, and the sedimented cells were resuspended at a density of 1×10^6 mL^{-1} in 5% FBS-supplemented RPMI 1640 medium. The cell suspension was added to each well of a 6- or 96-well tissue culture plate. After 2 hr of incubation in a humidified 5% CO_2 atmosphere at 37°, the medium was removed and cells were washed with PBS to remove those that were nonadherent. The required concentration of sanguiin H-6 or aminoguanidine with 10 μg/mL of LPS was then added to each well. The plates were incubated in a humidified 5% CO_2 atmosphere at 37° for 24 hr. Aliquots of the medium were used for determination of NO and cell viability. Nitrite was used as an indicator of NO production and was measured by a microplate assay method based on the Griess reaction [16]. Cell viability was estimated by the MTT assay [17]. For the assay of cell lysates, the cells were washed three times with PBS, scraped into cold PBS, and centrifuged at 500 *g* for 10 min at 4°. The cell pellet obtained was resuspended in 0.5 mL of 40 mM Tris buffer (pH 8) containing 5 μg/mL of pepstatin A, 1 μg/mL of chymostatin, 5 μg/mL of aprotinin and 100 μM phenylmethylsulfonyl fluoride, and then was lysed by three freeze–thaw cycles. Aliquots of the lysate were used for the determination of iNOS activity, and for protein assay. iNOS activity was measured as described previously [18]. Briefly, 10–20 μg of cell lysate protein was incubated in 20 mM Tris–HCl (pH 7.9), containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM DTT, and 2 mM each of L-Arg and NADPH. The reaction was carried out in duplicate for 180 min at 37° in 96-well plates. Residual NADPH was oxidized enzymatically with 10 U/mL of LDH and 5 mM sodium pyruvate in a final volume of 130 μL, by incubating for a further 5 min at 37°, and the Griess assay was performed as above. Protein was determined by the micro-biuret method [19], with bovine serum albumin as a standard.

2.5. RT-PCR analysis of iNOS mRNA

Macrophages (1×10^6 cells) were cultured with 10 μg/mL of LPS and sanguiin H-6 for 24 hr. Total RNA was extracted from the cell pellet with isogen according to the manufacturer's instructions. The RT reaction mixture (10 μL), containing 5 μg of total RNA, 2 μL of RT buffer (5×), 1 mM dNTP mix, 20 mM DTT, 1 μM oligo-dT primer, 2 U of RNase inhibitor and 200 U of RNase H-reverse transcriptase, was incubated at 42° for 60 min. The reaction was stopped by heating at 70° for 10 min. RT-generated cDNA encoding the iNOS and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (as an internal standard) genes was amplified using PCR. The iNOS primers (sense 5′-CAACCAGTATTATGGCTCCT-3′, antisense 5′-GTGACAGCCCGGTCTTTCCA-3′) and GAPDH primers (sense 5′-CCAAGGTCATCCATGACAAC-3′, antisense 5′-TTACTCCTTGGAGGCCCATGT-3′) were purchased from Nissinseihun. The 10-μL reaction mixture contained 1 μL of PCR buffer (10×), 2.2 mM $MgCl_2$, 0.25 mM dNTP mix, 0.25 μL each of iNOS and GAPDH cDNA primers, 1.25 U of recombinant Taq DNA polymerase, and 0.5 μL of RT product. The reaction consisted of 1 cycle at 94° for 5 min and then 30 cycles (1 min denaturation at 94°, 1 min annealing at 54°, and a 2-min extension at 72°) on a programmable thermal controller (GeneAmp™ PCR, System 9600, Perkin-Elmer). The PCR products were subjected to 3% agarose gel electrophoresis and stained with ethidium bromide. The luminescence intensity of the DNA bands was measured using a Bio-Rad densitometer (model GS-670) with Molecular Analyst software.

2.6. Scavenging effect on sodium nitroprusside-released NO radical

According to the method of Rao [20], sodium nitroprusside (5 mM) in PBS was mixed with different concentrations of sanguiin H-6 dissolved in 50 mM phosphate buffer, before being incubated at 25° for 150 min. The amount of NO produced by sodium nitroprusside was assayed by measuring the accumulation of nitrite, using a microplate assay method based on the Griess reaction.

2.7. Statistics

Results were assessed by analysis of variance, followed by Dunnett's test. Significance was accepted at $P < 0.05$.

3. Results

3.1. Effect of sanguiin H-6 on nitrite production in macrophages

Stimulation of macrophages with LPS elicited the accumulation of 49.86 μM nitrite, the stable end-product of NO, in medium, and this concentration of endotoxin reduced the cell viability (74.9% of untreated normal cells). In contrast, the untreated normal cells had only a small amount of nitrite accumulation (4.55 μM). When

Table 1
Effect of sanguiin H-6 on NO production in macrophages

Group	Nitrite (μM)	Cell viability (%)
None	4.55 ± 0.34	100 ± 1.3
LPS treatment		
Control	49.86 ± 1.44[c]	74.9 ± 2.4[c]
Sanguiin H-6 (12.5 μM)	15.60 ± 0.50[c,e]	82.9 ± 3.6[c,d]
Sanguiin H-6 (25 μM)	12.08 ± 0.96[c,e]	94.7 ± 1.3[a,e]
Sanguiin H-6 (50 μM)	7.75 ± 0.49[c,e]	107.6 ± 3.9[b,e]
Aminoguanidine (50 μM)	11.72 ± 0.53[c,e]	76 ± 2.7[c]

Statistical significance: [a]$P < 0.05$, [b]$P < 0.01$, and [c]$P < 0.01$ vs. non-treatment values; [d]$P < 0.01$, and [e]$P < 0.001$ vs. LPS treatment control values. Values are mean ± SEM, $N = 6$.

macrophages were cultured with LPS in addition to different concentrations of sanguiin H-6, the nitrite accumulation was inhibited substantially in a concentration-dependent manner, as shown in Table 1. The inhibition induced by 50 μM aminoguanidine resembled that found with 25 μM sanguiin H-6. However, it was determined that the cytotoxic effect of LPS could be attenuated by incubation with sanguiin H-6, with the cell viability improving significantly. Although aminoguanidine did improve cell viability to a minor extent, it appeared to have no ameliorative effect on the cytotoxicity caused by LPS (Table 1).

3.2. Effect of sanguiin H-6 on the expression of iNOS mRNA in macrophages

After a 24-hr incubation, iNOS mRNA expression was detected by electrophoretic analysis of the PCR products, and the results are shown at the top of Fig. 2. Unstimulated macrophages expressed little iNOS mRNA. In contrast, LPS stimulation induced a large amount of iNOS mRNA expression. Addition of sanguiin H-6 significantly suppressed the iNOS mRNA expression induced by LPS. The optical density scanning and normalized results of the electrophoretic photographs (the bottom graph in Fig. 2) showed that iNOS mRNA expression in the LPS-stimulated control was about 13-fold higher than in unstimulated macrophages, whereas in the presence of 12.5, 25, and 50 μM sanguiin H-6 this remarkable increase in the amount of iNOS mRNA was decreased to approximately 50, 24, and 17% of the LPS-stimulated control value, respectively.

3.3. Effect of sanguiin H-6 on enzyme activities of iNOS

A low level of iNOS activity was detected in LPS-unstimulated macrophages (5.87 pmol/mg protein/min), and this was increased about 4.4-fold, up to 25.98 pmol/mg protein/min by LPS stimulation, as shown in Table 2. Sanguiin H-6 inhibited the iNOS activity induced by LPS stimulation. This inhibitory effect was relatively weak at 12.5 μM, with a decrease of about 23%. However, the inhibitory effect significantly increased to 62% at a concentration of 25 μM, and to 73% at 50 μM.

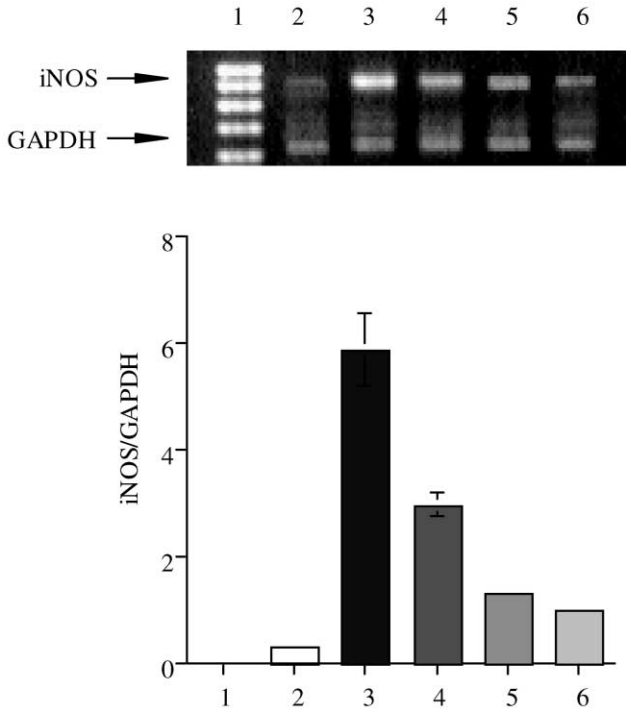

Fig. 2. Effect of sanguiin H-6 on iNOS mRNA expression in activated macrophages. (1) 50 bp marker DNA; (2) no treatment (control); (3) LPS-treated control; (4) LPS-treated sanguiin H-6 (12.5 μM); (5) LPS-treated sanguiin H-6 (25 μM); and (6) LPS-treated sanguiin H-6 (50 μM). Values are mean ± SEM, $N = 6$.

3.4. Effect of sanguiin H-6 on NO generation from sodium nitroprusside

Using the NO donor sodium nitroprusside, a significant amount of nitrite was formed by the reaction of oxygen with the NO produced from sodium nitroprusside. However, sanguiin H-6 inhibited this formation in a concentration-dependent manner, as shown in Table 3. NO production was only 4.78 μM when the concentration of sanguiin H-6 was increased to 100 μM.

Table 2
Effect of sanguiin H-6 on iNOS enzyme activity

Group	iNOS (pmol/mg protein/min)
None	5.87 ± 0.96
LPS treatment	
Control	25.98 ± 3.65[b]
Sanguiin H-6 (12.5 μM)	19.98 ± 2.72[b,c]
Sanguiin H-6 (25 μM)	9.80 ± 0.75[a,c]
Sanguiin H-6 (50 μM)	7.01 ± 1.10[c]
Aminoguanidine (50 μM)	9.75 ± 0.61[a,c]

Statistical significance: [a]$P < 0.05$, and [b]$P < 0.001$ vs. non-treatment values; [c]$P < 0.001$ vs. LPS treatment control values. Values are mean ± SEM, $N = 6$.

Table 3
Effect of sanguiin H-6 on NO generation from sodium nitroprusside

Sample	Concentration (μM)	NO (μM)[a]	Percent inhibition[b]
Sanguiin H-6	2.5	8.29 ± 0.07	37
	5	8.16 ± 0.09	37.9
	12.5	8.07 ± 0.10	38.6
	25	7.69 ± 0.07	41.5
	50	6.91 ± 0.10	47.5
	100	4.78 ± 0.05	63.7
Control	–	13.15 ± 0.11	

Values are mean ± SEM, $N = 5$.

[a] All treated NO values were statistically significant ($P < 0.001$) vs. control values.

[b] Percent inhibition = $A/B \times 100$ (A: individual value of NO production obtained in each group; B: control value).

4. Discussion

NO has various functions, and it exerts undesirable effects as well as physiologically important ones. In this connection, various attempts have been made to suppress the undesirable actions of NO, while leaving its favorable actions intact (or even facilitating them). Since the unfavorable actions of NO are attributable mainly to the NO produced by iNOS, a number of inhibitors for the suppression of iNOS induction or specific inhibition of its enzymatic activity have been developed. Many of these inhibitors are analogues of the substrate L-Arg. On the other hand, glucocorticoids such as dexamethasone, which are known to have a variety of actions, are effective in suppressing the induction of iNOS [21–23], although they are not specific for NOS.

Sanguiin H-6, the component examined in the present study, is the hydrolyzed tannin isolated from the crude drug Sanguisorbae Radix [24,25]. The present study found that this compound not only inhibited the expression of iNOS mRNA in a concentration-dependent manner, but also inhibited iNOS activity, demonstrating for the first time that a compound of this kind can inhibit the iNOS activity mediated by iNOS mRNA. However, the inhibitory mechanism of sanguiin H-6 remains to be fully elucidated. More specifically, it still remains unclear whether sanguiin H-6 inhibits the induction of iNOS mRNA by a direct action on LPS, or indirectly through the production/release of cytokines, where it could act on the signal transduction pathways involved in cytokine production by tyrosine kinases, or alternatively inhibit the phosphorylation of proteins induced by the cytokines themselves. Although the expression of iNOS mRNA and the iNOS activity were suppressed more as the concentration of sanguiin H-6 was increased, the production of NO was suppressed markedly even at a low concentration of this agent, suggesting the possibility that sanguiin H-6 directly eliminated NO. In another experiment using the NO donor sodium nitroprusside, sanguiin H-6 even at a low concentration was found to eliminate NO. These findings suggest that sanguiin H-6 has the capacity to directly eliminate NO and to suppress the iNOS gene-mediated system. However, the precise mechanism remains to be determined by further study.

The efficacy of aminoguanidine, an iNOS-selective inhibitor, in endotoxin shock has been reported [26]. Sanguiin H-6 at a concentration of 25 μM showed an effect equivalent to that of 50 μM aminoguanidine. Aminoguanidine resulted in no improvement in cell viability, which decreased in the presence of LPS, whereas sanguiin H-6 improved cell viability in a concentration-dependent manner, reducing the toxicity of LPS. Moncada *et al.* [1] have shown that the iNOS expressed in inflammatory cells produces a large amount of NO, and this not only acts as an effector for the non-specific defense mechanism, but also possibly damages normal cells, serving as an effector for autocytoclasis in autoimmune disease. Therefore, the ideal NOS inhibitor should not affect the favorable actions of NO and possibly may enhance them, blocking only the harmful actions. Currently, the available findings on sanguiin H-6 suggest that this agent has such an ideal activity. Although the exact mechanism has not been clarified, it may be a promising approach for the development of a safe selective iNOS inhibitor.

According to Narita *et al.* [27], in a renal injury model mediated by the immune reaction NO is the mediator of mesangial fusion, and inhibition of the NO production can reduce glomerular injury, which leads to subsequent glomerulosclerosis and tubular interstitium damage. Thus, it has been suggested that in renal injury mediated by the immune reaction, inhibition of the L-Arg/NO system at the initial stage may be a useful therapeutic measure. Although clarification of the effects of sanguiin H-6 on renal failure requires further investigation, it has become apparent that Sanguisorbae Radix extract significantly improves the high levels of NO and deteriorated renal function after LPS administration, and, in addition, it inhibits the iNOS activity in renal tissue. It is also known that Sanguisorbae Radix extract not only eliminates peroxynitrite but also reduces oxidative injury in the kidney [28–30], suggesting the importance of sanguiin H-6 in the treatment of renal injury. With this in mind, we intend to carry out further investigations on the role of sanguiin H-6 *in vivo*.

Acknowledgment

This work was supported, in part, by grants from the Japan Foundation for Aging and Health.

References

[1] Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109–42.

[2] Nathan C. Nitric oxide as a secretory product of mammalian cells. FASEB J 1992;6:3051–64.
[3] Kerwin Jr. JF, Lancaster Jr. JR, Feldman PL. Nitric oxide: a new paradigm for second messengers. J Med Chem 1995;38:4343–62.
[4] Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. J Biol Chem 1994;269:13725–8.
[5] Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J 1994;298:249–58.
[6] Ischiropoulos H, Zhu L, Beckman JS. Peroxynitrite formation from macrophage-derived nitric oxide. Arch Biochem Biophys 1992;298: 446–51.
[7] Stuehr DJ, Marletta MA. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. Proc Natl Acad Sci USA 1985;82:7738–42.
[8] Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J Biol Chem 1994;269:4705–8.
[9] Griffiths MJD, Messent M, MacAllister RJ, Evans TW. Aminoguanidine selectively inhibits inducible nitric oxide synthase. Br J Pharmacol 1993;110:963–8.
[10] Szabó C, Southan GJ, Thiemermann C. Beneficial effects and improved survival in rodent models of septic shock with *S*-methylisothiourea sulfate, a potent and selective inhibitor of inducible nitric oxide synthase. Proc Natl Acad Sci USA 1994;91:12472–6.
[11] Yokozawa T, Chen CP, Tanaka T. Direct scavenging of nitric oxide by traditional crude drugs. Phytomedicine 2000;6:453–63.
[12] Yokozawa T, Chen CP. Evidence suggesting a nitric oxide-scavenging activity for traditional crude drugs, and action mechanisms of Sanguisorbae Radix against oxidative stress and aging. J Am Aging Assoc 2001;24:19–30.
[13] Yokozawa T, Chen CP, Tanaka T, Kitani K. A study on the nitric oxide production-suppressing activity of Sanguisorbae Radix components. Biol Pharm Bull 2000;23:717–22.
[14] Sanguisorbae Radix. In: Jiangsu New Medical College, Edition. Chinese materia medica dictionary. Shanghai: Shanghai Science & Technique Press, 1987. p. 806–9.
[15] Lefkowitz SS, Brown DJ, Grattendick K, Lefkowitz DL. Cocaine inhibits production of murine hepatitis virus by peritoneal macrophages *in vitro*. Proc Soc Exp Biol Med 1997;215:87–93.
[16] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal Biochem 1982;126:131–8.
[17] Scudiero DA, Shoemaker RH, Paull KD, Monks A, Tierney S, Nofziger TH, Currens MJ, Seniff D, Boyd MR. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res 1988;48:4827–33.
[18] Suh N, Honda T, Finlay HJ, Barchowsky A, Williams C, Benoit NE, Xie QW, Nathan C, Gribble GW, Sporn MB. Novel triterpenoids suppress inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. Cancer Res 1998;58:717–23.
[19] Itzhaki RF, Gill DM. A micro-biuret method for estimating proteins. Anal Biochem 1964;9:401–10.
[20] Rao MNA. Nitric oxide scavenging by curcuminoids. J Pharm Pharmacol 1997;49:105–7.
[21] Radomski MW, Palmer RM, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc Natl Acad Sci USA 1990;87:10043–7.
[22] Rees DD, Cellek S, Palmer RMJ, Moncada S. Dexamethasone prevents the induction by endotoxin of a nitric oxide synthase and associated effects on vascular tone: an insight into endotoxin shock. Biochem Biophys Res Commun 1990;173:541–7.
[23] Pittner RA, Spitzer JA. Endotoxin and TNFα directly stimulate nitric oxide formation in cultured rat hepatocytes from chronically endotoxemic rats. Biochem Biophys Res Commun 1992;185:430–5.
[24] Nonaka G, Tanaka T, Nita M, Nishioka I. A dimeric hydrolyzable tannin, sanguiin H-6 from *Sanguisorba officinalis* L.. Chem Pharm Bull (Tokyo) 1982;30:2255–7.
[25] Nishioka I. Chemistry and biological activities of tannins. Yakugaku Zasshi 1983;103:125–42.
[26] Wolff DJ, Lubeskie A. Aminoguanidine is an isoform-selective, mechanism-based inactivator of nitric oxide synthase. Arch Biochem Biophys 1995;316:290–301.
[27] Narita I, Border WA, Ketteler M, Noble NA. Nitric oxide mediates immunologic injury to kidney mesangium in experimental glomerulo-nephritis. Lab Invest 1995;72:17–24.
[28] Chen CP, Yokozawa T, Kitani K. Beneficial effects of Sanguisorbae Radix in renal dysfunction caused by endotoxin *in vivo*. Biol Pharm Bull 1999;22:1327–30.
[29] Chen CP, Yokozawa T, Tanaka T. Protective effect of Sanguisorbae Radix against apoptosis and function of renal tissues subjected to ischemia-reperfusion. J Tradit Med 1999;16:97–101.
[30] Chen CP, Yokozawa T, Sekiya M, Hattori M, Tanaka T. Protective effect of Sanguisorbae Radix against peroxynitrite-mediated renal injury. J Tradit Med 2001;18:1–7.