Increase in nitric oxide synthase and cyclic GMP in vascular smooth muscle cells by treatment with aqueous extracts of Astragali Radix, Ginseng Radix and Scutellariae Radix

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(Received October 28, 1994. Accepted January 19, 1995.)

Abstract

Aqueous extracts of Astragali Radix, Ginseng Radix and Scutellariae Radix were previously shown to induce nitric oxide (NO) synthesis in cultured rat vascular smooth muscle cells (VSMC). The mechanism for this was investigated. Northern blot analysis showed that treatment with aqueous extracts caused increase in the transcription of inducible NO synthase (iNOS) gene in VSMC. Immunohistochemical and immunoblot analyses demonstrated significant increase of iNOS protein in VSMC after treatment with aqueous extracts.

These extracts increased dose-dependently intracellular cGMP in VSMC. This increase was inhibited by N^G-monomethyl-L-arginine, an inhibitor of NOS, or methylene blue, an inhibitor of soluble guanylate cyclase (sGC), indicating intracellular cGMP increase to be due to stimulation in the NO-sGC pathway. Aqueous extracts of Astragali Radix, Ginseng Radix and Scutellariae Radix can thus modulate cellular functions of VSMC by way of NO-sGC-cGMP mediated signal transduction pathways.

Key words nitric oxide, nitric oxide synthase, rat vascular smooth muscle cells, cyclic GMP, Astragali Radix, Ginseng Radix, Scutellariae Radix.

Abbreviations NO, nitric oxide ; NOS, nitric oxide synthase ; iNOS, inducible nitric oxide synthase ; cNOS, constitutive nitric oxide synthase ; VSMC, vascular smooth muscle cell ; cGMP, guanosine 3',5'-cyclic monophosphate ; sGC, soluble guanylate cyclase ; EDRF, endothelium-derived relaxing factor ; EDTA, ethylenediaminetetraacetate ; SDS, sodium dodecyl sulfate ; PAGE, polyacrylamide gel electrophoresis ; L-NMA, N^G-monomethyl-L-arginine ; MB, methylene blue ; PBS, phosphate buffered saline.

Introduction

Nitric oxide (NO) is a multifunctional mediator with various important physiological functions such as neurotransmission, non-specific immune defence and vasodilation.[1] In the vasculature, NO was originally identified as the endothelium-derived relaxing factor (EDRF) and has been shown essential to the control of vascular tone and peripheral blood flow.[2,3] Endothelium-derived NO relaxes vascular smooth muscle cells(VSMC) and inhibits platelet adhesion and aggregation.[2,3] VSMC not only responds to endothelium-derived NO but also possesses enzymes to produce NO from L-arginine.[4] Charpie *et al.* found VSMC-derived NO to function as an autocrine factor in the regulation of vascular tone.[5] These features of NO are attributed to its ability to activate soluble guanylate cyclase(sGC) and thus increase intracellular guanosine 3',5'-cyclic monophosphate (cGMP).[2]

Aqueous extracts of Astragali Radix, Ginseng Radix and Scutellariae Radix were previously shown

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和漢医薬学雑誌 12, 38－44, 1995

to induce NO generation in clutured rat VSMC.[6] The mechanism for this was investigated in this study. Treatment with aqueous extracts induced transcription of the inducible NO synthase (iNOS) gene with consequent intracellular accumulation of iNOS protein. NO-dependent accumulation of cGMP was also demonstrated in VSMC after treatment with extracts. Aqueous extracts of Astragali Radix, Ginseng Radix and Scutellariae Radix thus modulate the cellular function of VSMC by way of NO-sGC-cGMP mediated signal transduction pathways.

Materials and Methods

Cell preparation and cultures : VSMC were isolated from the thoracic aortas of 200 - 300 g male Sprague-Dawley rats by enzymatic dissociation as described previously.[6] The cells were grown in RPMI1640 medium supplemented with 10 % heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. They were passaged by harvesting with trypsin/EDTA, and cells in subculture passage 10-20 were used for all experiments. Cells were identified as smooth muscle cells based on the characteristic "hill and valley" growth pattern and staining wih a monoclonal antibody specific for α-SMC-actin.

Preparation of aqueous extracts of crude drugs : All crude drugs (medicinal plants) were purchased from Uchida Wakan-Yaku Co. Ltd. (Tokyo, Japan). The crude drugs, cut into small pieces, were suspended in 20 volumes distilled water and gently boiled until the solution was reduced by a half volume. The extracts were filtered while warm and lyophilized. The lyophilized extracts were weighed and re-suspended in distilled water at 20 mg/ml to obtain the stock solutions. The ratios of yield of aqueous extracts obtained by this method were 22 % (Astragali Radix), 22 % (Ginseng Radix) and 20 % (Scutellariae Radix) of original dry weight of crude drugs.

Treatment with aqueous extracts of crude drugs : The cells were seeded into 48-well plates for cGMP assay or into 6-well plates for RNA preparation at a cell density of 6×10^5/ml, and pre-incubated for 24 hr before the treatment. For experimental treatment, cells were exposed for 24 hr to each of aqueous extracts of crude drugs by adding concentrated stock solutions into the culture media. The dosages of the extracts used in this study were 500 μg / ml for Astragali Radix and Ginseng Radix, and 100 μg/ml for Scutellariae Radix. These dosages were previously shown to be effective on the induction of NO synthesis in VSMC.[6] After incubation in culture media with herbal extracts for 24 hr, cells were subjected to analyses for intracellular levels of iNOS protein, iNOS mRNA and cGMP as follows.

Sodium dodecyl sulfate polyacrylamide gel elctrophoresis(SDS-PAGE) and immunoblot analysis : SDS-PAGE and immunoblot analysis were performed as described previously.[7] Briefly, cell lysates matched for cell number (2×10^5 cells/lane) were subjected to SDS-PAGE in a 8 % polyacrylamide slab gel. Separated proteins were transferred to a nitrocelluolose membrane and iNOS protein was detected with a monoclonal antibody specific to mouse macrophage iNOS (Transduction Laboratories, Lexington, KY).

Northern blot hybridization : Total RNA was extracted from VSMC by a modified guanidinium thiocyanate method using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacture. Northern blot hybridization was carried out essentially as described by Sambrook *et al.*[8] Briefly, samples 10 μg each total RNA were denatured and subjected to electrophoresis on a 1 % agarose-formaldehyde gel.

After electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination to verify even loading and determine ribosomal RNA positions. RNAs were transferred to Hybond-N nylon membranes (Amersham Life Science, Tokyo, Japan) and fixed by UV irradiation. Each membrane was hybridized with ^{32}P-labeled iNOS cDNA probe. After hybridization, the membranes were washed under high-stringency conditions. The washed blot was autoradiographed using Kodak XAR-5 film with an intensifying screen at -70°C.

The probe was a 1033-bp cDNA fragment of mouse macrophage iNOS gene prepared by the reverse transcription polymerase chain reaction method from LPS-stimulated RAW264 cells according to Koide *et al.*[9]. The sequences of the forward (ACAGGGAAGTCTGAAGCACTAG) and reverse

(CATGCAAGGAAGGGAACTCTTC) primers were used to amplify the 1033-bp cDNA fragment of mouse iNOS cDNA sequence (nucleotides 1621-2653).[9] The iNOS cDNA fragment was labeled with [α-^{32}P] dCTP by the randam priming method. The cDNA fragment shared 92 % nucleotide sequence homology with the corresponding fragment of iNOS cDNA cloned from cultured rat VSMC.[10] iNOS mRNA was thus determined in rat VSMC on cross-species hybridization with the mouse iNOS cDNA fragment.

Immunohistochemistry : VSMC were grown on chambered glass slides (Nunc Inc., Naperville, IL) to subconfluence and cultured for 24 hr in the presence or absence of herbal extracts. The cells were fixed with 0.1 M phosphate buffer (pH 7.4) containing 4 % paraformaldehyde and treated sequentially with 0.3 % hydrogen peroxide in 100 % methanol to quench endogenous peroxidase activity, 0.3 % Triton-X100 to permeabilize the fixed cells and 10 % normal goat serum to block nonspecific staining. The cells were incubated with 10 μg / ml monoclonal anti mouse macrophage iNOS antibody (Transduction Laboratories, Lexington, KY). After several washings in phosphate buffered saline (PBS), they were incubated with biotin-conjugated horse anti-mouse IgG (Vector Laboratories, INC., Burlingame, CA). After further washing in PBS, the cells were treated for 30 min with streptavidin conjugated with horseradish peroxidase (Vector Laboratories, INC.). Specifically bound antibodies were visualized by a peroxidase substrate kit DAB (Vector Laboratories, INC.)

cGMP measurement : The medium was aspirated, and the cells were immediately scraped from the dish with 6 % trichloroacetic acid. The cell suspension was sonicated for 5 seconds and centrifuged at 2000 × g for 15 minutes at 4°C to separate precipitated proteins. The supernatants were extracted four times with 5 volumes water-saturated diethylether to remove trichloroacetic acid in the supernatant. The amount of cGMP in each sample was determined using a commercially available cGMP EIA kit (Amersham Life Science,Tokyo), with an acetylation step added to increase sensitivity. Intracytoplasmic cGMP was expressed in femtomoles per 10^4 cells.

Results

Transcriptional induction of the iNOS gene in VSMC

To determine the effects of aqueous extracts on transcription of the iNOS gene, expression of iNOS mRNA was assessed by Northern blot hybridization using ^{32}P-labeled mouse iNOS cDNA as a probe.

As shown in Fig. 1, iNOS mRNA was barely detectable in total RNA prepared from unstimulated VSMC (lane 1), Intracelluar iNOS mRNA markedly increased at 24 hours after treatment with aqueous extracts of Ginseng Radix, Astragali Radix or Scutellariae Radix (lanes 2-4).

Intracytoplasmic iNOS protein levels in VSMC

Changes in iNOS protein in VSMC were evaluated by immunohistochemical staining and immunoblot analysis using a monoclonal antibody specific to mouse macrophage iNOS protein. As shown in Fig.

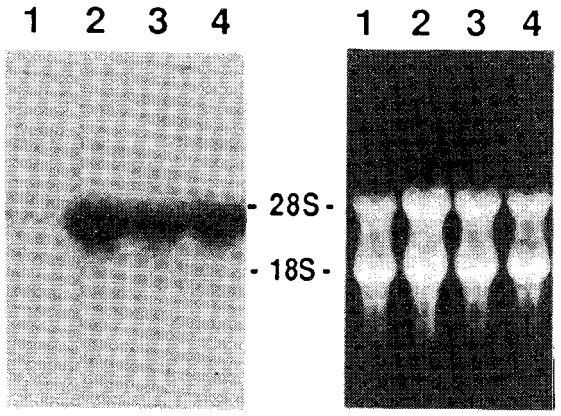

Fig. 1 Northern blot analysis showing the effects of aqueous extracts of Ginseng Radix, Astragali Radix and Scutellariae Radix on iNOS mRNA in cultured VSMC. Ten μg total RNAs isolated from VSMC cultured for 24 hr in control medium (lane 1), or presence of aqueous extracts of Ginseng Radix (lane 2), Astragall Rasix (lane 3), or Scutellariae Radix (lane 4) were separated by 1 % agarose-formaldehyde gel electrophoresis following Northern blot hybridization using ^{32}P labeled mouse iNOS cDNA probe. An autoradiograph of Northern blot analysis showing the expression levels of iNOS mRNA appears on the left. A photograph of the ethidium bromide stained gel prior to transfer of RNAs to the nylon membrane is shown on the right to demonstrate even loading and positions of ribosomal RNAs. The dosages of each extract are as follows : Ginseng Radix, 500 μg / ml ; Astragali Radix, 500 μg/ml ; Scutellariae Radix, 100 μg/ml.

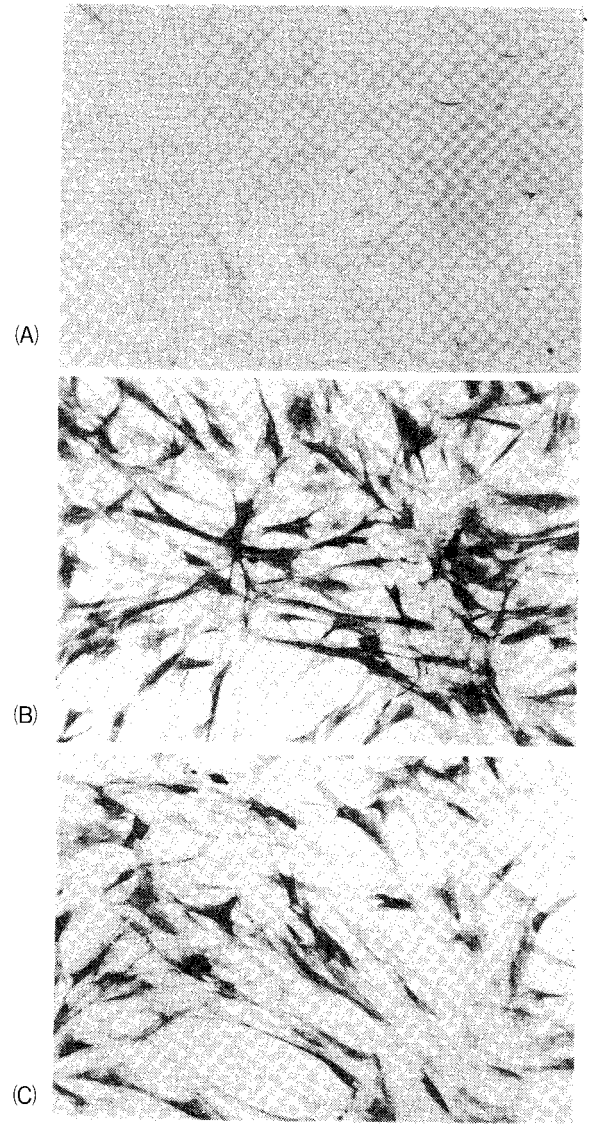

Fig. 2 Immunohistochemical staining of iNOS protein in VSMC cultured for 24 hr in the absence (A), or presence of aqueous extract of Astragali Radix (B) or Ginseng Radix (C). The dosages of each extract are the same as Figure 1. Basal amount of iNOS protein in the untreated VSMC was immunologically undetectable (A). Staining of iNOS, shown as dark brown, was clearly evident in VSMC after treatment with aqueous extracts of the crude drugs (B and C). Positive signals are distributed in the cytoplasm.

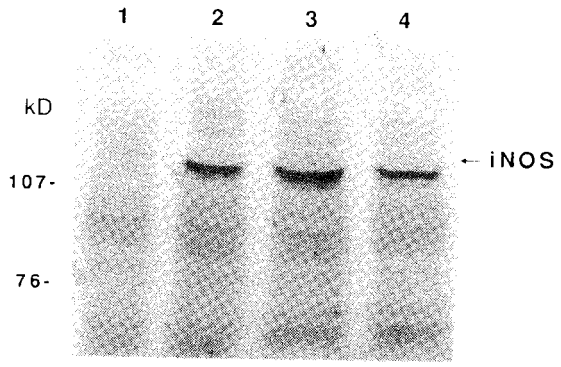

Fig. 3 Immunoblot analysis showing intracytoplasmic amount of iNOS protein in VSMC cultured for 24 hr in the control medium (lane 1), or presence of aqueous extracts of Ginseng Radix (lane 2), Astragali Radix (lane 3) or Scutellariae Radix (lane 4). The dosages of each extract are the same as Figure 1. The immunoreactive band of iNOS is barely detectable in the control culture (lane 1). The iNOS protein band with a molecular mass of 130 kDa appeared after exposure to aqueous extracts of Astragali Radix, Ginseng Radix, or Scutellariae Radix (lane 2–4).

2A, basal iNOS protein in the untreated VSMC was immunologically undetectable. On the other hand, staining for iNOS was clearly evident in VSMC after treatment with aqueous extracts of Astragali Radix (Fig. 2B) or Ginseng Radix (Fig. 2C). Similar positive staining for iNOS was observed in VSMC treated with Scutellariae Radix (data not shown). Consistent results were obtained by immunoblot analysis as shown in Fig. 3. In unstimulated cells, the immunoreactive band of iNOS was barely detectable (Fig. 3, lane 1). The iNOS protein band with a molecular mass of 130 kDa appeared after exposure to aqueous extracts of Astragali Radix, Ginseng Radix, or Scutellariae Radix (Fig. 3, lane 2–4).

cGMP in VSMC

Basal intracytoplasmic cGMP in VSMC were less than 3 fmol per 10^4 cells. However, there was significant and dose-dependent increase in cGMP 24 hr after treatment with aqueous extracts of Astragali Radix, Ginseng Radix or Scutellariae Radix (Fig. 4). Fig. 5 shows the effects of N^G-monomethyl-L-arginine (L-NMA) and methylene blue on increase in cGMP in VSMC induced by aqueous extracts of crude drugs. Increase in intracellular cGMP induced by treatment with the herbal extracts was significantly reduced by simultaneous incubation with 1mM L-NMA or 5 μM methylene blue. L-NMA and methylene blue are inhibitors of NOS and soluble guanylate cyclase (sGC), respectively. The present result thus indicates increase in cGMP by aqueous extracts of these crude drugs to be due to stimulation in the NO sGC pathway.

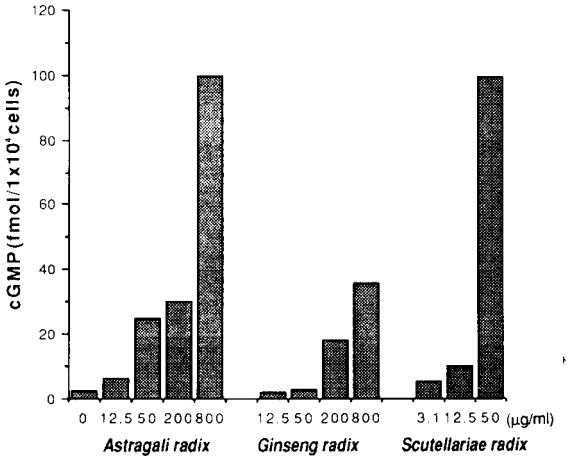

Fig. 4 Measurement of cGMP in VSMC cultured for 24 hr in the absence (control) or presence of aqueous extracts of Astragali Radix, Ginseng Radix or Scutellariae Radix. The amount of cGMP of each sample was determined using a commercially available kit, with an acetylation step added to increase sensitivity. Intracytoplasmic cGMP is expressed in femtomoles per 10^4 cells. Data shown are representative of three independent experiments, which gave similar results. Values represent means of two samples with duplicate measurements.

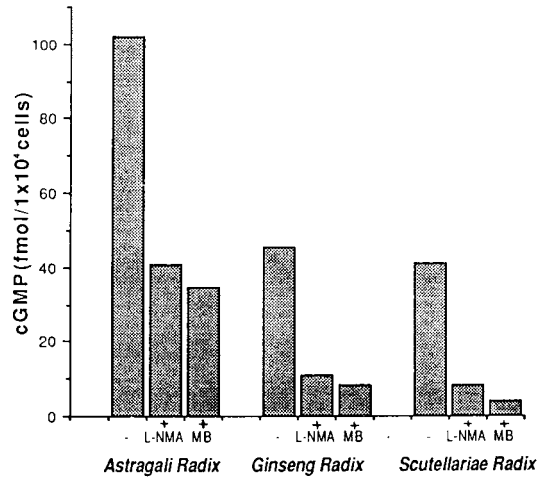

Fig. 5 Effects of N^G-monomethyl L-arginine (L-NMA) and methylene blue (MB) on cGMP production in VSMC induced by aqueous extracts of Astragali Radix, Ginseng Radix or Scutellariae Radix. The dosages of each extract are the same as Figure 1. Increase in intracelluar cGMP induced by herbal extracts was markedly reduced by the simultaneous incubation with 1 mM L NMA or 5 μM MB. Intracytoplasmic cGMP is expressed in femtomoles per 10^4 cells. Data shown are representative of three independent experiments, which gave similar results. Values represent means of two samples with duplicate measurements.

Discussion

NO plays a fundamental role in the control of blood pressure and regional blood flow through activation of sGC and resultant increase in cGMP in VSMC.[1-3] Aqueous extracts of Ginseng Radix, Astragali Radix and Scutellariae Radix were previously shown to increase NO production in cultured VSMC.[6] In this study, treatment with these extracts resulted in transcriptional induction of the iNOS gene with consequent increase in intracytoplasmic iNOS protein in VSMC. The intracellular accumulation of cGMP was noted due to increase in NO production. It thus follows that aqueous extracts of crude drugs modulate signal transduction mediated by the NO-sGC-cGMP pathway in VSMC. Thus possibly, NO-mediated increase in cGMP in VSMC may be a mechanism by which crude drugs exhibit vasodilation, inhibition of platelet adhesion and aggregation and anti-atherosclerotic effects.[11,12]

NO is derived from terminal guanido-nitrogen of L-arginine by inducible or constitutive isoforms of NO synthase (NOS). Constitutive NOS (cNOS) in endothelial cells generates low levels of NO in response to hormones or sheer stress.[3] Some crude drugs show vasodilatory action by way of stimulation in the endothelium-derived NO production.[13,14] Inducible NOS (iNOS) is not present under basal conditions, but is expressed in response to cytokines or bacterial endotoxin in macrophages and VSMC.[3] iNOS generates substantially larger amounts of NO for longer periods of time, and conspicuous NO produced by iNOS in VSMC has been implicated in several pathological states including septic shock in which the overexpression of NO results in significant cardiovascular dysfunction.[15,16] A sepsis-related decrease in blood pressure is considered due at least in part to the vasodilating action of NO synthesized in VSMC by iNOS induced by endotoxin, TNF-α and IL-1.[15,16] The functional consequences of the induction of VSMC-derived NO by herbal extracts may thus be a basis for determining whether the effects are beneficial or harmful to cardiovascular physiology.

NO possibly has many anti-atherogenic properties.[2] These include inhibition of the adhesion and

aggregation of platelets, superoxide anion production, leukocyte adhesion, and VSMC proliferation. Hoggs *et al.* found NO to inhibit LDL oxidation by acting as a chain-breaking antioxidant.[17] The oral administration of L-arginine, the nitric oxide precursor, showed anti atherogenic effects in hypercholesterolemic rabbit and inhibited development of intimal hyperplasia following balloon catheter-induced injury.[18,19] NO may thus act to inhibit atherosclerotic lesions.

If the degree of NO production in VSMC by herbal extracts is moderate compared to that by IL-1β or LPS, and does not reach pathological levels that would cause severe vascular dysfunction, VSMC-derived NO may improve regional blood flow or prevent atherosclerosis. Although the actual physiologic and pathologic significance of NO production in VSMC induced by these herbal extract remains to be elucidated, this paper shows for the first time that aqueous extracts of Astragali Radix, Ginseng Radix and Scutellariae Radix modulate the NO-sGC-cGMP-mediated signal transduction pathway in VSMC *in vitro*.

Although we demonstrated a possible mechanism for NO induction in VSMC by crude drugs, it is still difficult to elucidate the active component(s) for the effects. This is mainly due to the complexity of ingredients of crude drugs. For example, we previously reported that baicalein, a flavone of Scutellariae Radix, inhibits NO synthesis in LPS-stimulated macrophages.[20] This finding, together with the results in the present study, indicates that an aqueous extract of Scutellariae Radix contains both inhibitory and stimulatory components for NO synthesis. We recently find that some kinds of polysaccharides may be involved in the NO induction from VSMC (Kido, T. *et al.* unpublished data). Elucidation of active component (s) for NO induction in VSMC may contribute to the quality control of these crude drugs in relation to their pharmacological effects in VSMC. Further studies should be conducted to determine the *in vivo* effects of these crude drugs on the NO-sGC-cGMP pathway in the cardiovascular system.

和文抄録

前報において，人参，黄耆，黄芩の各水エキスがラット培養血管平滑筋細胞（VSMC）に対して，一酸化窒素（NO）産生刺激作用を示すことを報告した。今回その作用メカニズムを明らかにする目的にて，細胞内の誘導性NO合成酵素（iNOS）の蛋白量を免疫組織染色とWestern blot法にて，mRNAレベルをNorthern blot法にて検討した。これら3種の生薬水エキスはラットVSMCに対して，iNOS遺伝子の転写を誘導し，細胞内iNOS蛋白量を増加させることが明らかとなった。さらに，NOによるguanylate cyclase（GC）の活性化を介した細胞内cGMPの増加も認めた。以上の結果により，人参，黄耆，黄芩の各水エキスが，VSMCのNO-GC-cGMPを介したシグナル伝達系に作用する可能性を報告した。

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