Vol. 214, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 5, 1995 Pages 145-151

N^{ω}-HYDROXYAGMATINE: A NOVEL SUBSTANCE CAUSING ENDOTHELIUM-DEPENDENT VASORELAXATION

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Received July 29, 1995

Summary: Agmatine (Agm), a decarboxylated L-arginine, has been suggested to be an endogenous clonidine-displacing substance in the brain. We hypothesed that Agm enzymatically produces nitric oxide (NO) through N^{ω}-hydroxyagmatine (OHAgm) similar to the well-known endogenous NO generation from L-arginine through N^{ω}-hydroxy-L-arginine, because Agm possesses a guanidyl function in its molecule. OHAgm was originally synthesized from δ-aminopentanoic acid in 36 % overall yield. Agm and the synthetic OHAgm were examined using rat aortic rings whether they could cause endothelium-dependent vasorelaxation or not. These substances equally elicited vasorelaxations. The relaxations were completely abolished by a NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester, or endothelium denudation. These results suggested that Agm and OHAgm are alternative precursors for NO generated by NOS and that OHAgm may be an endogenous substance distributable in the brain.

Nitric oxide (NO) plays important biological roles in cardiovascular system, central and peripheral nervous systems, and immune system (1). It is well known that NO is biosynthesized *in vivo* during a two step oxidation of L-arginine (Arg) into L-citrulline (Cit) through N^{ω}-hydroxy-L-arginine (OHArg) by a NO synthase (NOS). In this pathway a key function is a guanidyl group of Arg (2, 3). Recently Reis *et al.* reported that agmatine (Agm), a decarboxylated Arg, was an endogenous clonidine-displacing substance (CDS) in the brain (4). Clonidine has been used as an antihypertensive drug and contains a modified guanidyl function in its molecule. We hypothesed that (i) Agm could enzymatically generate NO by oxygenation of the guanidyl group of Agm through N^{ω}-hydroxyagmatine (OHAgm), similar to the well-known endogenous NO generation from Arg through OHArg (4), and (ii) the generated NO might be partly responsible for the biological action due to CDS in the brain. Thus, we tested Agm and OHAgm on endothelium-dependent vasorelaxation in order to examine whether these act as precursors of NOgeneration or not.

In this report we present (i) a synthetic procedure of OHAgm from δ-aminopentanoic acid and the characterization of this newly synthesized substance, (ii) the endothelium-dependent vasorelaxation

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0006-291X/95 $12.00

caused by Agm and the synthetic OHAgm, and (iii) the possibility of OHAgm as a biosynthetic intermediate in an altenative pathway of NO generation from Agm.

Materials and Methods

Chemicals: All commercially available chemicals were purchased from Nacalai tesque (Kyoto, Japan) or Aldrich Chemical Company, Inc. (Milwaukee, USA) and generally used without further purification. Tetrahydrofuran (THF) was purified by refluxing with sodium and benzophenone, and dioxane was distilled in the presence of sodium. Silica gel No 7734 (Merck; Darmstadt, Germany) and silica gel 60 (Nacalai) were used for column chromatography and flash chromatography (6), respectively.

Synthetic studies: OHAgm was synthesized through five intermediates as shown in Fig 1 and the synthetic procedures were described as follows. (i) *N*-[t-Butoxycarbonyl (Boc)]-δ-aminopentanoic acid: δ-Aminopentanoic acid (0.103 g, 0.88 mM) was dissolved in 10 % triethylamine (TEA) in MeOH (13 ml) and the solution was added to di-t-butyl dicarbonate (0.375 g, 0.17 mM) with vigorously stirring. The reaction mixture was stirred at 40 °C for 1 day. After evaporation of the solvent under reduced pressure EtOH (1 ml) and 5 % NaOH aq (1.5 ml) were added to the residue, respectively and the whole was stirred at 75°C for 3 h. After addition of water the neutral component was removed by extraction with Et_2O. The aqueous layer was acidified with 5 % HCl aq (pH 3-4) and extracted with Et_2O. The ethereal solution was dried over $MgSO_4$, and evaporated to dryness under reduced pressure. Recrystallization of the crude product from hexane-Et_2O afforded *N*-[t-butoxycarbonyl (Boc)]-δ-aminopentanoic acid (0.169 g, 89 % yield), mp 49-51 °C [mp 49-51 °C (7)], as colorless prisms. (ii) *N*-Boc-*N'*-benzyloxycarbonyl (Cbz)-1,4-diaminobutane: To a mixture of *N*-Boc-δ-aminopentanoic acid (0.861 g, 3.96 mM) and TEA (1.0 ml, 9.92 mM) in THF (20 ml) was added isobutyl chloroformate (1.0 ml, 7.9 mM) followed by a solution of sodium azide (4.76 g, 73.7 mM) in H_2O (16 ml) at 0 °C. The reaction mixture was stirred at 0°C for 7 h and extracted with ethyl acetate. The ethyl acetate solution was washed with H_2O, dried over K_2CO_3, and evaporated to dryness under reduced pressure. The crude acyl azide (1.09 g) was dissolved in benzene (57 ml) and the solution was stirred at 70 °C overnight. After addition of further benzene (60 ml), benzyl alcohol (4 ml, 38. 5 mM) and *p*-toluenesulfonic acid monohydrate (0.08 g, 0.42 mM), the mixture was stirred at rt for 3.5 h, washed with 10 % Na_2CO_3 aq and water, and then dried over K_2CO_3. After evaporation of the benzene purification of the crude product by column chromatography using hexane : Et_2O=1 : 2 gave *N*-Boc-*N'*-Cbz-1,4-diaminobutane as colorless fine needles (1.01 g, 80 % yield), a part of which was recrystallized from hexane-Et_2O to afford colorless fine needles, mp 103-104 °C. IR (JASCO, IR-700) ν_{max} ($CHCl_3$) cm^{-1}: 3458 (NH), 1709 (CO); ^1H NMR [JOEL, JNM GXS-500α (500 MHz, in $CDCl_3$)] δ 1.41 (9H, s, t-Bu), 1.53 (4H, br s, 2-, 3-H_2), 3.13 (2H, br s, 4- H_2), 3.21 (2H, q like, *J*=7.4 Hz, 1-H_2), 4.57 (1H, s, NH, exchangeable), 4.83 (1H, s, NH, exchangeable), 5.10 (2H, s, OCH_2Ph), 7.25-7.40 (5H, m, OCH_2Ph); Anal. Calcd for $C_{17}H_{26}N_2O_4$: C, 63.33; H, 8.13; N, 8.69. Found: C, 63.41; H, 7.94; N, 8.64; HRFABMS (JOEL, JMX-MX 110A) *m/z*: 323.1952 (Calcd for $C_{17}H_{27}N_2O_4$: 323.1971). (iii) *N*-Boc-1,4-diaminobutane: A solution of *N*-Boc-*N'*-Cbz-1,4-diaminobutane (1.04 g, 3.24 mM) in EtOH (63 ml) containing 10 % palladium on carbon (Pd-C) (0.104 g) was hydrogenated at rt and at atomspheric pressure for 2 h to give *N*-Boc-1,4-diaminobutane as a labile colorless oil (0.647 g, 100 % yield), which was used for the following reaction without further purification. IR ν_{max} ($CHCl_3$) cm^{-1}: 3456 (NH), 1705 (CO); ^1H NMR (500 MHz, in $CDCl_3$) δ 1.41 (9H, s, t-Bu), 1.45-1.60 (2H, br s, 2- or 3-H_2), 1.60-1.70 (2H, br s, 4-H_2), 3.03-3.70 (2H, br s, 1-H_2), 4.90-5.20 (3H, br s, NH, NH_2, exchangeable). (iv) *N*-Boc-*N'*-cyano-1,4-diaminobutane: A solution of bromocyan (95 %, 0.257 g, 2.3 mM) in anhydrous MeOH (0.8 ml) was added to a solution of the crude *N*-Boc-1,4-diaminobutane (0.364 g, 1.9 mM) in anhydrous MeOH (6.4 ml) in the presence of anhydrous sodium acetate (0.311 g, 3.8 mM). The mixture was stirred at the same temperature for 2.5 h. After evaporation of the MeOH the residue was suspended to Et_2O and the insoluble material was removed, and the filtrate was evaporated under reduced pressure. Purification of the crude oil by column chromatography using hexane : ethyl acetate=1 : 10 afforded a cyanamide as a labile colorless oil (0.341 g, 88 % yield). IR ν_{max} ($CHCl_3$) cm^{-1}: 3454 (NH), 2224 (CN), 1707 (CO); ^1H NMR

(500 MHz, in $CDCl_3$) δ 1.44 (9H, s, t-Bu), 1.56-1.60 (2H, m, 2- or 3-H_2), 1.60-1.70 (2H, m, 3- or 2-H_2), 3.10-3.20 (4H, m, 1-, 4-H_2), 4.05, 4.63 (each 1H, s, NH, exchangeable). (v) *N*-Boc-*N'*-hydroxyagmatine: A mixture of the cyanamide (0.083 g, 0.38 mM), hydroxylamine hydrochloride (0.058 g, 0.83 mM), and TEA (0.06 ml, 39.5 mM) in EtOH (1.1 ml) was stirred at rt for 1.3 h. After addition of H_2O the mixture was extracted with ethyl acetate. The organic solution was dried over K_2CO_3 and evaporated to dryness. Purification of the crude oil by column chromatogarphy using $CHCl_3$: MeOH=5 : 1 afforded *N*-Boc-*N'*-hydroxyagmatine as a colorless oil (0.055g, 57 % yield). IR ν_{max} ($CHCl_3$) cm^{-1}: 3452 (NH), 3500-3200 (OH), 1707 (CO); ^1H NMR {JOEL JNM GXS-400α [400 MHz, in $CDCl_3$+CD_3OD (1 drop)]} δ 1.43 (9H, s, t-Bu), 1.50-1.52 (4H, m, 2-, 3-H_2), 3.04 (2H, t, *J*=6.6 Hz, 1-H_2), 3.10 (2H, t, *J*=7.1 Hz, 1-H_2); ^{13}C NMR [125 MHz, in $CDCl_3$+CD_3OD (1 drop)] δ 26.77 (2-CH_2), 27.27 (3-CH_2), 28.33 (C(*C*H_3)3), 39.83 (1-CH_2), 41.11 (4-CH_2), 77.20 (*C*(CH_3)3), 156.09 (CO), 156.38 (C=N); HRFABMS *m/z*: 247.1771 (Calcd for $C_{10}H_{23}N_4O_3$: 247.1770). (vi) OHAgm: The protected OHAgm (0.02g, 0.085 mM) was dissolved in dioxane (1.0 ml) saturated with dry hydrogen chloride gas and the mixture was kept to stand at rt for 1h. OHAgm was separated as a yellow viscous mass and collected by decantation quantitatively. ^1H NMR (500 MHz, in CD_3OD) δ 1.63-1.80 (4H, m, 2-, 3-H_2), 2.97 (2H, q, *J*=7.5 Hz, 4-H_2), 3.27 (2H, t, *J*=7.0 Hz, 1-H_2); ^{13}C NMR [125 MHz, in CD_3OD] δ 24.78 (3-CH_2), 25.78 (2-CH_2), 39.81 (4-CH_2), 41.17 (1-CH_2), 159.54 (C=N); FABMS *m/z*: 147.1246 (Calcd for $C_5H_{15}N_4O$: 147.1238).

Biological assays: Male Wister rats (250-300 g) were used in the studies. The thoracic aorta was excised and cut into 3 mm width of rings. Intact or endothelium denuded rings were suspended in 10 ml tubes filled with Krebs' bicarbonate solution (120 mM NaCl, 5.2 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.03 mM Na_2EDTA, and 11 mM dextrose: pH 7.4 at 37 °C), and were treated with 1 mM N^{ω}-nitro-L-arginine methyl ester (NAME) in half of aortic rings. Then, these were precontracted with 100 nM norepinephrine (NE), and were subjected to test the relaxation against Amg or OHAgm (10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M). The changes of isometric tension were recorded. In other experiments, acetylcholine (Ach) was cumulatively applied to test whether Agm and OHAgm enhance the endothelium dependent vascular relaxation. The denudation of endothelium and/or the inhibition of NOS were confirmed by Ach-induced vascular relaxation prior to each study.

Results

Chemical studies: OHAgm was prepared from δ-aminopentanoic acid as shown in Fig 1. The amino group of δ-aminopentanoic acid was firstly protected with a Boc group. The Curtius rearrangement of *N*-Boc-δ-aminopentanoic acid followed by treatment with benzyl alcohol afforded *N*-Boc-*N'*-Cbz-1,4-diaminobutane as a crystalline mass. The Cbz protecting group of the diprotected diaminobutane was selectively removed by catalytic hydrogenation with 10 % Pd-C to yield an oily mono Boc-protected diaminobutane. Treatment of the diamine with bromocyan gave a cyanamide as a labile oil. Functionalization of the cyanamide with hydroxylamine hydrochloride afforded a Boc-protected OHAgm. Removal of the Boc group of a protected OHAgm with hydrogen chloride-saturated dioxane afforded a desired OHAgm as a yellow viscous mass.

The overall yield of OHAgm from δ-aminopentanoic acid was 36 %. All synthetic compounds including OHAgm were characterized by IR, NMR, and/or MS spectra. OHAgm ($C_5H_{14}N_4O$) expectedly showed a protonated molecular ion peak at *m/z*: 147.1246 (Calcd for $C_5H_{15}N_4O$: 147.1238) in the HRFABMS spectrum. The ^1H and ^{13}C NMR spectra of OHAgm were given in Fig 2. In the ^1H NMR spectrum a 4H singlet-like signal due to 2- and 3-CH_2 group appeared at δ 1.70

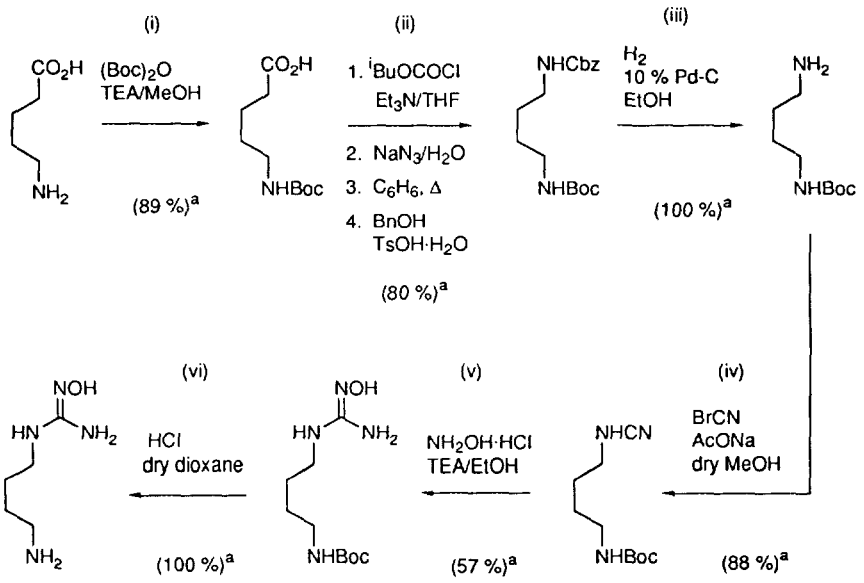

Fig. 1. Synthesis of OHAgm from δ-aminopentanoic acid. [a] A value in the parentheses is a chemical yield in each step.

ppm, and two 2H triplets due to 1- and 4-CH_2 one appeared at δ 3.02 and 3.27 ppm, respectively. These signals were reasonably observed in the ^{13}C NMR spectrum, in which the hydroxyguanidyl carbon additionally appeared at δ 159.5 ppm.

Biological assays: The endothelium denudation and/or the NOS inhibition by NAME were confirmed by the complete abolishment of Ach-induced vascular relaxation (Table 1). Both Agm and OHAgm relaxed the intact aortic rings in a dose-dependent manner; the tension was decreased by 81± 8 (mean ±SD) % and 74±16 % of the NE-induced precontraction at the concentration of 10^{-5} M,

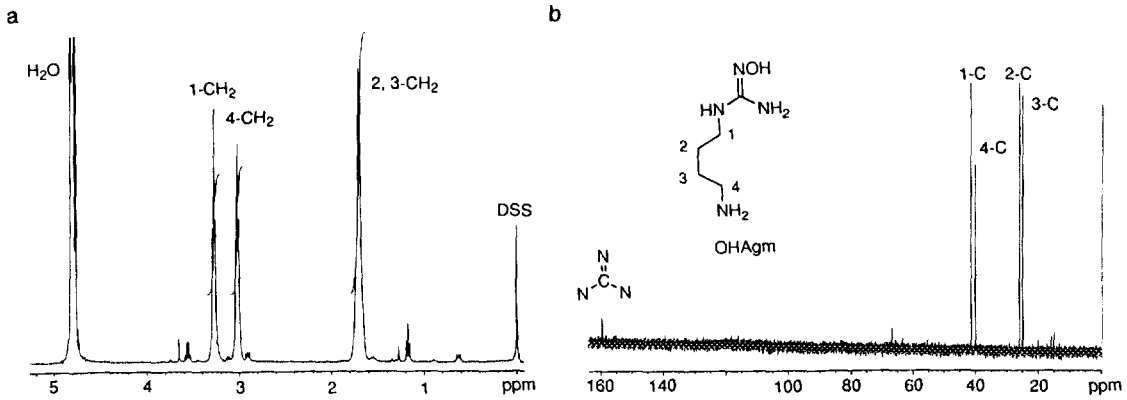

Fig. 2. The charts of the ^1H NMR (500 MHz, in D_2O) (a) and the ^{13}C NMR (125 MHz, in D_2O) (b) spectra of OHAgm.

Table 1. The confirmation of endothelium denudation and NOS inhibition used in the biological assays

Treatment		Isometric tension [a]	
Endothelium	NAME	NE (10^{-7} M)	Ach (10^{-7} M)
intact	10^{-3} M	25.5 ± 6.9	34.3 ± 11.8
intact	no treatment	17.7 ± 5.9	1.0 ± 1.0
denuded	10^{-3} M	23.5 ± 5.9	29.4 ± 9.8
denuded	no treatment	23.5 ± 6.9	25.5 ± 7.8

[a] The rat aorta was contracted with NE (10^{-7} M) and the Ach (10^{-7} M)-induced relaxation was tested. The values were isometric tension (10^{-2} dyn) and mean ± SD of 25 assays.

respectively (Fig 3). Neither of them, however, relaxed the endothelium-denuded rings. The NOS inhibition by NAME also abolished Agm- and OHAgm-induced vascualr relaxation completely. There were no differences between those responses to Agm and OHAgm. The Ach-induced endothelium-dependent vascular relaxation was, however, not changed by pretreatment with either Agm or OHAgm at the concentration of 10^{-5} M (Fig 4).

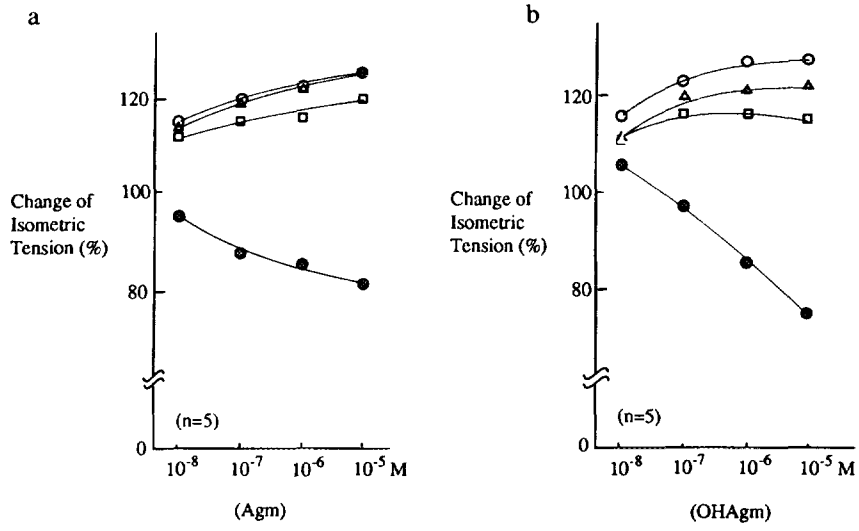

Fig.3. Effects of Agm (a) and OHAgm (b) on vascular tone. The rat aortic rings were contracted with 10^{-7} M NE, and Agm or OHAgm was cumulatively added on. The ordinates are changes of isometric tension against NE-induced precontraction.
●: Endothelium (+), NAME (-) □: Endothelium (-), NAME (-)
○: Endothelium (+), NAME (+) △: Endothelium (-), NAME (+)

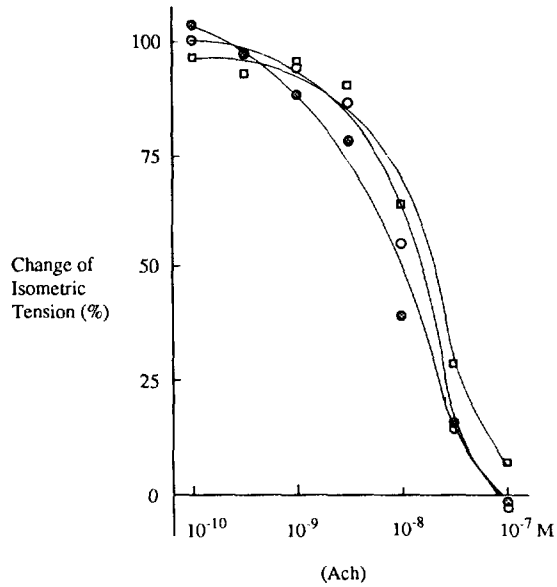

Fig.4. Effects of Agm or OHAgm on Ach-induced vascular relaxation. The intact aortic rings treated with Agm or OHAgm were contracted with 10^{-7} M NE, and Ach was cumulatively added on. The ordinate is a change of isometric tension against NE-induced precontraction.

○ : control (n=5) □ : Agm (10^{-5}M) (n=5) ◎ : OHAgm (10^{-5}M) (n=5)

Discussion

In 1984, Altas and Burstein partially purified CDS from calf brain, which was not a catecholamine nor a peptide and had an estimated mass of 520 daltons (8, 9). Recently, Reis *et al.* reported that CDS was Agm, which was an endogenous agonist at imidazoline receptors, a noncatecholamine ligand at α_2-adrenagic receptors and may act as a neurotransmitter (3). However, soon later Altas argued against the conclusion by Reis *et al.* and claimed that the CDS and Agm were not the same because of the different molecular and physiological properities of these compounds, although the structure of CDS was not still elusive at this point (10).

Our results showed that both Agm and OHAgm caused vasorelaxation, which was inhibited by endothelium denudation or by NOS inhibition. Although there has remained discussion on whether CDS is Agm or not (11), these facts may support the possibilities of the physiological action due to Agm in the brain in spite of our incomplete result depending on endothelium-dependent vasorelaxation activity in smooth muscle system.

Zembowicz *et al.* suggested that HOArg reacts chemically with NO released from bovine aortic endothelial cells, and forms a potent and more stable vasodilator which is most likely through a reaction with the hydroxyguanidino group (12). They examined the reaction of a hydroxyguanidino group with NO using a simpler molecule, hydroxyguanidine (HOG), and concluded that the interaction of the hydroxyguanidino group with NO was responsible for the vasorelaxation (13).

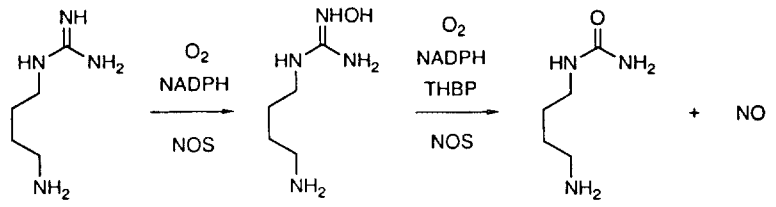

Fig.5. Hypothesis of NO generation from Agm through OHAgm.

If their conclusion was applied to interpret our results, the effect of OHAgm on vasorelaxation may simply attributable to the presence of the hydroxyguanidino group in its molecule. However, Agm, lacking a hydroxyguanidyl group, also showed the same endothelium-dependent relaxation as OHAgm did. These facts suggested that alternative pathway(s) responsible for the vasorelaxation should be present in endothelial cells, in additon to the possible pathway due to the reaction between a hydroxyguanidino group and NO. It would be reasonable to suppose that the vasorelaxation caused by Agm and OHAgm are attributable to NO, which can be generated by the similar biosynthetic pathway to that from Arg to Cit through OHArg (Fig 5). Thus, we conclude that Agm and OHAgm should be alternate precursors for the biosynthesis of NO and that OHAgm must be an endogenous substance causing endothelium-dependent vasorelaxation, although identification of OHAgm in tissues has remained.

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