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Bis-*N*-nitroso-caged Nitric Oxides: Photochemistry and Biological Performance Test by Rat Aorta Vasorelaxation

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Abstract—Three new caged nitric oxides (NOs)—BNN3, BNN5Na, and BNN5M—were tested for biological use. BNNs have a strong ultraviolet (UV) absorption band (λ_{max}: 300 nm, ε: 13.5 $mM^{-1}\,cm^{-1}$) extended to 420 nm and produce NO upon irradiation with 300–360 nm light in quantum yields about 2. A photoexcited BNN molecule yields two NOs with time constants of less than 10 ns for phase 1 and less than 20 μs for phase 2 at 37°C, suggesting usefulness of BNNs for measuring in vivo and in vitro fast NO reactions. Upon irradiating with UV light, caged nitric oxides-loaded rat aortic strips maintained in a state of active tonic contraction effectively relaxed (< 3 μM BNN5M loading solution concentration). BNN3 is incorporated in the lipid membrane. BNN5Na, insoluble in organic solvents but water soluble, localizes in the water phase. BNN5M, is muscle-cell-permeable and hydrolysed to BNN5Na to remain in cytosol. BNNs were thermally stable and demonstrated no observable toxicity.

Introduction

Nitric oxide (NO) plays diverse roles in mammals, e.g. in blood pressure control, platelet aggregation inhibition, neurotransmission, immune regulation, and penile erection.[1–7] NO is also considered to relate to redox signaling via *S*-nitrosothiol formation and decomposition.[8] To explore these biological activities, it would be desirable to create a high-performance caged NO that, when light irradiated, generated desired NO at specific target-tissue sites in a specific time. Photolytically generating bioactive molecules (uncaging) from photolabile precursors (caged compounds) is already generally used in the biosciences.[9] Although new types of photo-removable protective groups are being developed,[10–14] most caged compounds thus far are *o*-nitrobenzyl esters of bioactive target molecules.[9,15]

Ortho-nitrobenzyl caged NO (CNO1-4) developed by Makings and Tsien[15] was used to study the NO role as a messenger initiating long-term depression in cerebellum,[16] and long-term potentiation in hippocampus CA1 region[17] and cultured hippocampal neurons.[18] $Ru(NO)Cl_5$[19] and Rousin's salt,[20] an iron–sulfur nitrosyl cluster, were used similarly. *S*-nitrosothiols[21–23] and 2-methyl-2-nitrosopropane[24,25] were also reported to produce NO upon irradiation with light. However, quantum yields of uncaging for these known caged NOs are quite poor. Both Rousin's salt[26,27] and *S*-nitrosothiols[28] thermally produce NO without light, and further trace amounts of transition metal ion effectively catalyze NO extrusion from *S*-nitrosothiols.[29–31] So, the biological use of these two is limited.

Thus, we synthesized three different caged NOs (bis-*N*-nitroso compounds) based on a new molecular design (Fig. 1), i.e. *N*,*N*′-dinitroso-*N*,*N*′-dimethylphenylenediamine (BNN3), *N*,*N*′-dinitrosophenylenediamine-*N*,*N*′-

Abbreviations: NO, nitric oxide; BNN3, *N*,*N*′-dinitroso-*N*,*N*′-dimethylphenylenediamine; BNN5, *N*,*N*′-dinitrosophenylenediamine-*N*,*N*′-diacetic acid; BNN5Na, *N*,*N*′-dinitrosophenylenediamine-*N*,*N*′-diacetic acid sodium salt; BNN5M, dimethyl *N*,*N*′-dinitrosophenylenediamine-*N*,*N*′-diacetate; LFP, Laser flash photolysis; mp, melting point; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PBS, phosphate buffered saline solution; DMSO, dimethyl sulfoxide; $TPPCo^{II}$, meso-tetraphenylporphinatocobalt(II); ODQ, 1H-[1,2,4]oxazolo[4,3-a]quinozalin-1-one; carboxy-PTIO, 2-(4′-carboxyphenyl)-3,3,4,4-tetra-methylimidazoline-1-oxyl-3-oxide; PTIO, 2-phenyl-3,3,4,4-tetramethylimidazoline-1-oxyl-3-oxide.

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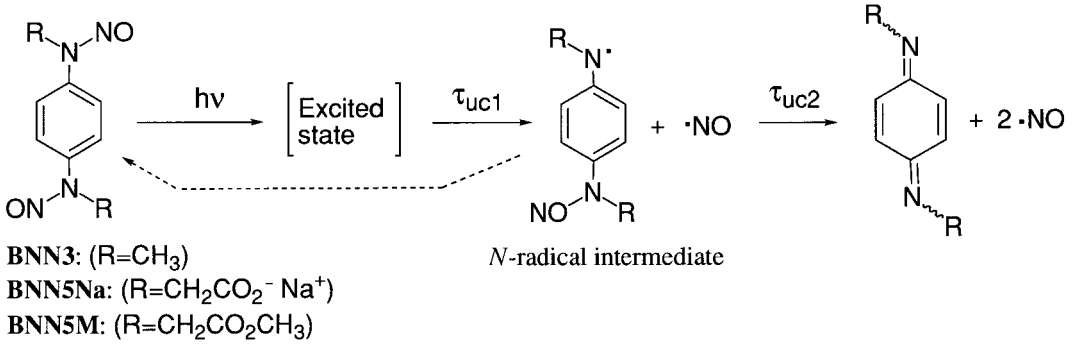


Figure 1. Uncaging of caged nitric oxides.

diacetic acid sodium salt (BNN5Na) and a BNN5 dimethyl ester (BNN5M). BNN3 is soluble in organic solvents but water-insoluble.[32] BNN5Na is water soluble but insoluble in organic solvents.[32] BNN5M dissolves well in organic solvents and is slightly water-soluble. Quantum yields of BNN3 and BNN5Na are almost 2, i.e. absorption of one photon by one BNN3 or BNN5Na molecule yields two NO molecules through an *N*-radical intermediate.[32] BNNs are thus high-performance in a chemical sense, but must be evaluated biologically before general biological application can be attempted.

Our purposes are to: (a) introduce a new BNN member, *N*,*N*′-dinitrosophenylenediamine-*N*,*N*′-diacetate (BNN5M), (b) obtain kinetic data on LFP of BNNs at 37°C, (c) test caged NOs performance for biological applications by rat aortic strips photorelaxation, and (d) detect the localisation of BNNs compartment. Vasorelaxation assay is suitable for testing caged NO performance, because a large number of vasorelaxation experiments with NO and NO-producing systems have been reported in an attempt to identify the chemical feature of endothelial-derived relaxant factor,[1–7,33] ever since Furchgott and Zawadzki suggested its presence.[34]

Results

Stability of caged NO

BNN5Na was found to be stable in 100 mM phosphate buffer of pH 7.4 at 37°C for over 1 day in the dark. When BNN5Na was incubated with 10 mM cysteine in phosphate buffer of pH 7.4 at 37°C for 2 h, no noticeable reaction occurred. When benzene solutions of BNN3 and BNN5M were kept at 37°C in the dark for 2 h, no noticeable reaction occurred.

Laser flash photolysis (LFP) of caged NOs

τ_{uc} is an important photochemical constant in uncaging kinetics, because τ_{uc} is the time constant for the unimolecular reaction of the photoexcited caged molecule with a first-order rate constant, k_{uc}, yielding the target bioactive molecule, i.e. the reciprocal of k_{uc}. Uncaging kinetics of BNN3 and BNN5Na at 23°C were previously studied using laser flash photolysis (LFP).[32] Given that caged NOs would be used in living systems, LFP experimental data at 37°C would be important. A 500 μM BNN5M solution has been laser-photolyzed with a 20 ns pulse width and 308 nm wavelength at 37°C. Because BNN5M is not sufficiently for water-soluble experiments, benzene solution was used. An absorbance decay curve was observed at 400 nm due to intermediary *N*-radicals (λ_{max} 400 nm). Absorbance recorded during the reaction from 5 to 50 μs after irradiaton fits first-order kinetics (simulation curve with rate constant, $k_{uc2} = 1.74 \times 10^5 s^{-1}$; Fig. 2(A)). During the initial 5 μs, *N*-radicals disappear, however, faster than the calculated rate based on the first order decay with the above k_{uc2} indicating a second-order reaction of the *N*-radical and NO to regenerate BNN5M.[32] The *N*-radical appeared within 10 ns after the laser flash, indicating that the excited state of BNN5M splits to yield the *N*-radical intermediate and the first NO molecule within 10 ns. The uncaging of BNN5M occurs with the τ_{uc1} < 10 ns for the first NO-generating phase and τ_{uc2} = 5.7 μs for the second phase. The same experiments

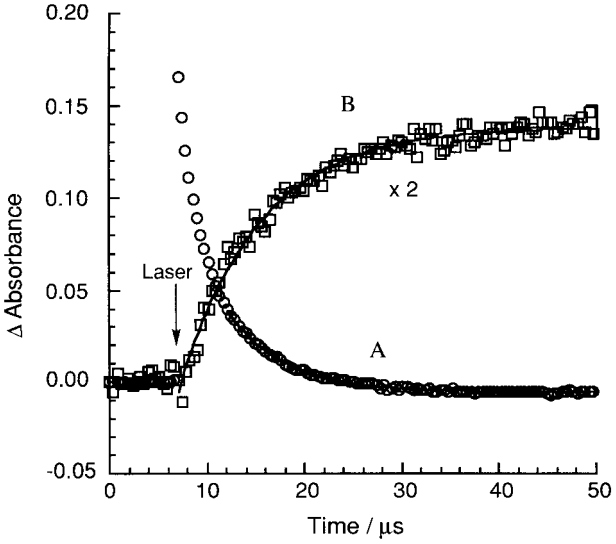


Figure 2. Laser flash photolysis of BNN5M in benzene at 37°C. (A): BNN5M alone; [BNN5M] = 100 μM; absorbance at 400 nm of *N*-radical intermediate traced. (B): Mixture of BNN5M and $TPPCo^{II}$; [BNN5M] = 12.3 μM; [$TPPCo^{II}$] = 52.1 μM; absorbance at 546 nm of $TPPCo^{II}$ NO complex traced. Solid lines are theoretical first-order kinetic curves.

for BNN5Na in an aqueous buffer solution at 37°C showed that $\tau_{uc1} < 10$ ns for the first phase and $\tau_{uc2} = 19$ μs for the second phase. Similarly for BNN3, $\tau_{uc1} < 10$ ns for the first phase and $\tau_{uc2} = 5.8$ μs for the second phase in benzene at 37°C.

To demonstrate the very small τ_{uc} values of BNN5M and its usefulness in exploring fast NO reaction kinetics, LFP of 12.9 μM BNN5M solution was conducted in the presence of 65.8 μM mesotetraphenylporphinatocobalt(II) ($TPPCo^{II}$), a model of soluble guanylate cyclase (Fig. 3), in benzene at 37°C. Raising absorbance at 546 nm is due to $TPPCo^{II}NO$ (Fig. 2(B)). Experimental points (Fig. 2) fit well to the first-order kinetic equation to obtain a pseudo-first-order rate constant, $1.32 \pm 0.03 \times 10^5$ s^{-1}, at 37°C. The same experiments were conducted using different $TPPCo^{II}$ concentrations (40–95 μM) at a constant BNN5M concentration (12.9 μM). From the slope of the linear plot of pseudo-first-order rate constants against $TPPCo^{II}$ concentrations (Fig. 4), the second-order rate constant for combination of $TPPCo^{II}$ and NO in benzene at 37°C was found to be $1.99 \pm 0.03 \times 10^9$ M^{-1} s^{-1}.

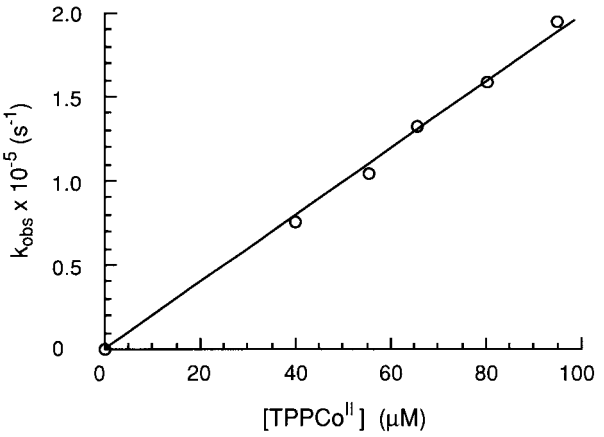


Figure 4. Plot of k_{obs} of NO binding to $TPPCo^{II}$ against $TPPCo^{II}$ concentration. LFP experiments were carried out with [BNN5M] = 12.9 μM and [$TPPCo^{II}$] = 40.1–95.1 μM in benzene under Ar at 37°C.

Caged NO photovasorelaxation assay

Endothelial cells-denuded rat aortic strips were incubated with one of the BNN reagents in an organ bath maintained at 37°C. After strips with BNN were loaded into the organ bath, the organ bath solution was replaced with fresh Krebs–Ringer's solution to remove free BNN molecules. After repetition of the same washing procedure, aortic strips were then irradiated using 300 or 360 nm UV light while recording strip tension. Furchgott et al. reported that UV light irradiation induces relaxation of the rabbit aorta, maintained in a state of active tonic contraction.[35–37] Some photosensitive NO donor seems to be inherently present in smooth muscles, but mechanisms remain controversial.[38–42] Taking this phenomenon into account, carefully controlled experiments have been conducted without using caged NOs, and irradiation of strips was found to cause substantial relaxation (Table 1, entries 1 and 6). Net BNN-induced photorelaxation was obtained by subtracting relaxation value of control experiments from that for BNN-loaded strips. As soon as the light-path shutter for uncaging was opened for photons to reach the aorta strip, quick relaxation occurred (Fig. 5). Reduction of the relaxation response during repeated uncaging is presumably due to the consumption of both incorporated caged NO and unknown photo-sensitive NO-donor inherently contained in smooth muscle cells.[36,42] The photoinduced vasorelaxation time course of the caged NO-loaded strips was similar to that caused by adding aqueous NO solution to the organ bath (data not shown). When the light-path shutter was closed, tension returned to the steady state initially induced by methoxamine (Fig. 5).

Oxyhemoglobin, a typical NO quencher in biological systems, and 1*H*-[1,2,4]oxazolo[4,3-*a*]quinoxalin-1-one (ODQ),[43] a guanylate cyclase inhibitor, inhibited the BNN5Na-dependent photoinduced vasorelaxation (Fig. 5(B) and (C)). Results of these inhibition tests reveal that the BNN5Na-dependent photoinduced vasorelaxation is caused by activating guanylate cyclase with NO formed by photolytic uncaging of BNN5Na.

Under all assay conditions, photoinduced vasorelaxation of BNN reagent-loaded strips was reversible and repeatable. Thus, at least for the contractile apparatus, BNN reagents show no cytotoxicity.

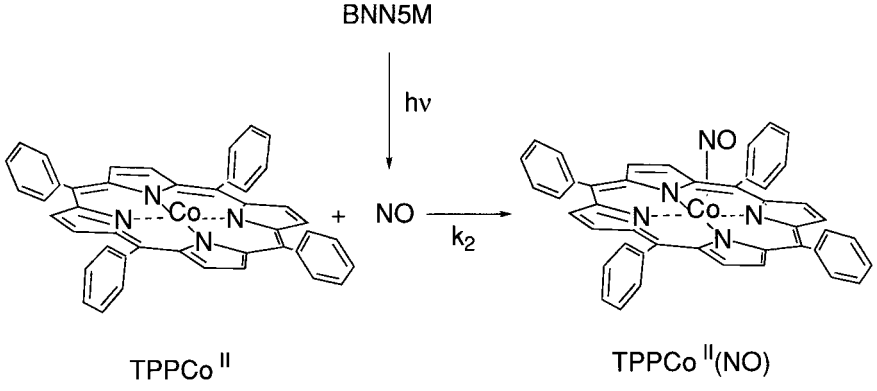


Figure 3. Uncaging of BNN5M in heme-model presence.

Table 1. Photorelaxation of caged NO-loaded rat aorta strips with 1 μM BNN for 30 min

Entry	Caged NO	Wavelength (nm)[c]	Total % relaxation[d]	BNN-induced % relaxation[a]
1[a]	Control	300[f]	28.3 ± 2.6[e]	0
2[a]	BNN3	300[f]	49.6 ± 3.1	30.1 ± 9.0
3[a]	BNN5Na	300[f]	35.4 ± 6.0	10.4 ± 12.3
4[b]	BNN5Na	300[f]	60.6 ± 8.7	45.7 ± 17.4
5[a]	BNN5M	300[f]	69.7 ± 2.5	58.1 ± 9.2
6[a]	Control	360[g]	46.8 ± 2.4[e]	0
7[a]	BNN5M	360[f]	60.0 ± 3.9	25.4 ± 13.0

[a] Photolysis was conducted in BNN-free Krebs–Ringer's solution after washing strips.
[b] Photolysis was conducted in Krebs–Ringer's solution containing 1 μM of BNN5Na.
[c] Wavelength of uncaging light (light path slitwidth, ±2 nm).
[d] $n = 4$.
[e] $n = 6$. Strips were not treated with caged NO.
[f] 0.26 mW.
[g] 0.45 mW.

In assay results for photoinduced vasorelaxation of the three caged compounds (Table 1), except for entries 1, 4, and 6, strips were rinsed with caged NO-free Krebs–Ringer's solution after loading of caged NOs. Data demonstrate three points;

a. In irradiation of rat aortic strips in the organ solution containing 1 μM BNN5Na, substantial relaxation was observed (entry 4). However, water-soluble BNN5Na did not cause vasorelaxation, when strips were rinsed with BNN5Na-free Krebs–Ringer's solution after treatment with BNN5Na (entry 3). Combining these two and the previous observation that the BNN5Na-dependent photoinduced vasorelaxation is inhibited by haemoglobin, one can say that BNN5Na does not penetrate smooth muscle cells through the plasma membrane but NO formed by the uncaging of BNN5Na present in the medium between the strip and the light-delivering optical fiber top penetrated tissue to induce vasorelaxation.[44]
b. Since BNN3 and BNN5M induced vasorelaxation upon uncaging even after strips were rinsed with caged NO-free Krebs–Ringer's solution to remove BNN5M from the medium (entries 2 and 3), BNN3 and BNN5M were cell-membrane permeable.
c. Control vasorelaxation induced by 360 nm light is 1.7-fold greater than that by 300 nm light (entries 1 and 6). This is because 360 nm light is 1.9-fold stronger than 300 nm light (see Experimental). The net BNN5M dependent vasorelaxaton caused by 300 nm light is 23-fold greater than that by 360 nm light (entries 5 and 7). Shorter wavelength light (300 nm) is somewhat effective for uncaging caged NO in rat aortic strips.

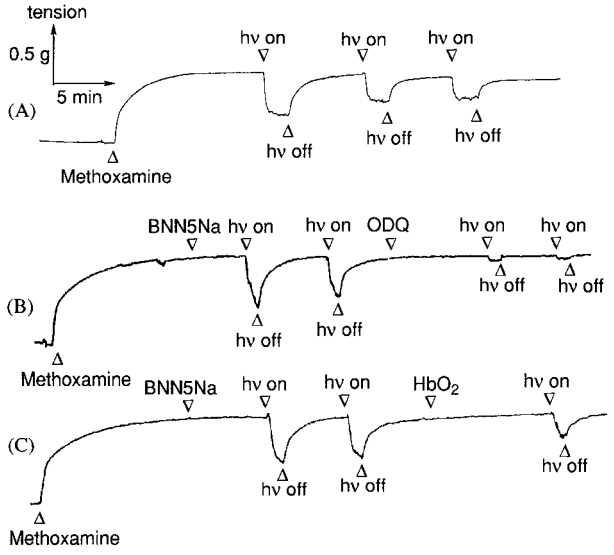


Figure 5. Some selected traces. (A) Typical photoinduced BNN5M-loaded rat aorta vasorelaxation ([BNN5M]: 1 μM; loading time: 30 min). After BNN5M doping, the strips were rinsed by displacing it twice in an organ bath solution with BNN5M-free Krebs–Ringer's solution. (B), (C) Inhibition of photoinduced vasorelaxation in 1 μM BNN5Na-containing Krebs–Ringer's solution with (B) ODQ; (C) 50 μM oxyhemoglobin (HbO_2).

Fixing the BNN5M concentration for the loading solution at 1 μM, we studied the effect of loading time on photorelaxation. Results (Fig. 6) showed that reagent incorporation into the cell proceeds very effectively. As

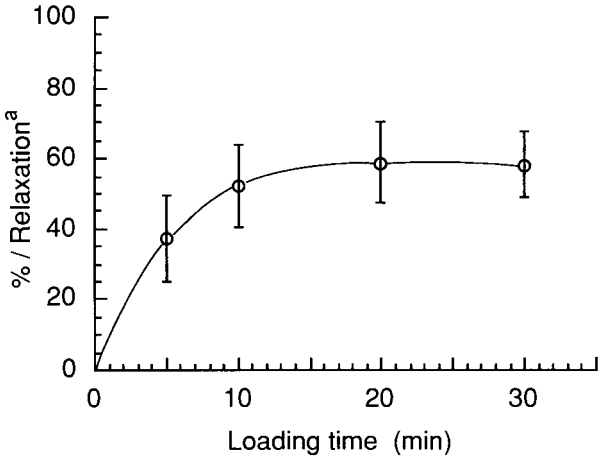


Figure 6. Effect of BNN5M-loading time on rat aortic strip photorelaxation. Incubation solution contained 1 μM BNN5M. After incubation, the strips were rinsed with fresh Krebs–Ringer's solution and irradiated with 300 nm wavelength light. $n = 4$. Error bars: SEM.

can be seen in the BNN5M concentration–vasorelaxation relationship (Fig. 7), strips treated with even 100 nM of BNN5M underwent 25% relaxation. The relaxation response increased concentration-dependently, and peaked (ca. 80%) to level off at about 3 μM BNN5M (Fig. 7).

BNN5M hydrolysis with muscle cells

Incubation of 0.75 mL solution containing 9.4 μM BNN5M and cytosolic fraction obtained from ca. 5×10^6 cultured muscle cells for 20 min at 37°C resulted in the hydrolysis of BNN5M to yield 5.5 μM BNN5Na. The result encouraged us to examine the possibility that BNN5M may enter cells and be hydrolyzed to yield BNN5Na (Fig. 8). A10 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 100 μM BNN5M at 37°C. After incubation at different time intervals, the cytosolic fraction was isolated and the BNN5Na content was measured (Fig. 9). The BNN5Na concentration in the cytosolic fraction increases with incubation time until about 40 min, then plateaued.[45]

Discussion

The chemical quality of a caged compound is expressed by physicochemical constants, $\varepsilon\Phi_{uc}$ and τ_{uc}, specific to each caged compound. ε is the molar absorption coefficient of the caged compound at the wavelength of uncaging light, and Φ_{uc} is the quantum yield of uncaging. $\varepsilon\Phi$ value is thus a parameter representing the relative amount of bioactive molecules generated by uncaging with light of a unit strength from unit amounts of caged compounds at a unit time interval. τ_{uc} is the time constant for the uncaging process of the photoexcited caged molecule yielding the target bioactive molecule. Thus, a chemically high-performance caged compound possesses a large $\varepsilon\Phi_{uc}$ and a small τ_{uc}.

In addition to these chemical requirements, high-performance caged compounds, which are useful in bioscientific application, should have the following biological properties: (a) the caged compound is itself bioinactive and (b) the caged compound is localized at the specific compartment at which the researcher wants to generate target bioactive molecules.

$\varepsilon\Phi_{uc}$ and vasorelaxation

Three BNN reagents have a strong UV absorption band extended to 425 nm wavelength (λ_{max}: 292–300 nm, ε: 13,500–13,700 M^{-1} cm^{-1}) and the chemical yields of NO in uncaging of both BNN5Na in water and BNN3

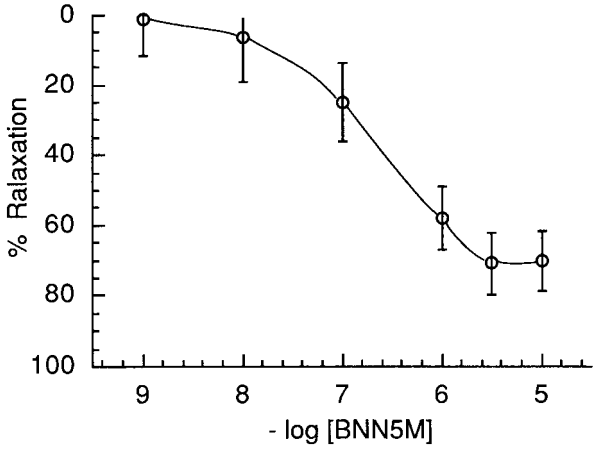


Figure 7. Influence of BNN5M loading solution concentration on rat aorta strip relaxation ($n=4$; loading time: 30 min at 37°C). Error bars: SEM.

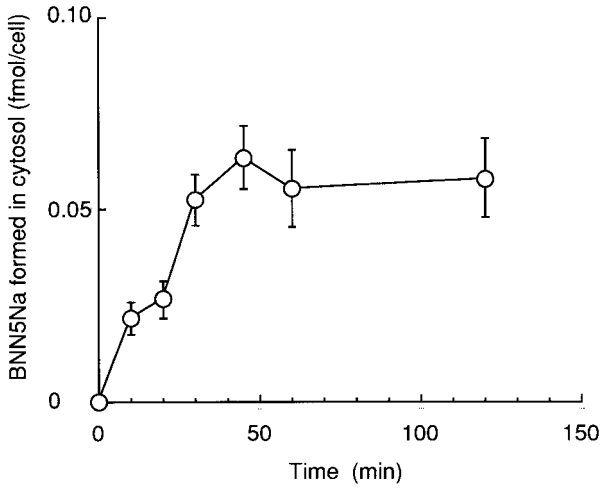
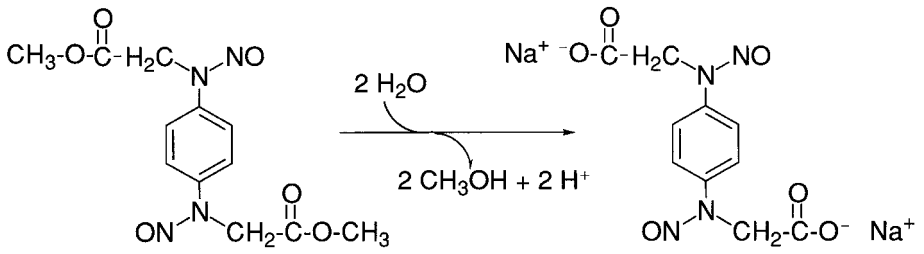


Figure 9. Time course for BNN5Na formation in smooth muscle cell cytosol fraction during BNN5M incubation with cultured smooth muscle cells. $n=4$. Error bars: SEM.



BNN5M **BNN5**

Figure 8. Enzymatic BNN5M hydrolysis.

in organic solvents are quantitative. When 2-(4-carboxyphenyl)-3,3,4,4-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) and 2-phenyl-3,3,4,4-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) are used as NO scavengers, the quantum yield for BNN5Na uncaging in water is 1.87 and that for BNN3 in organic solvents is 2, i.e. a single photon produces 1.6–2 NO molecules from one BNN molecule.[32] Values of Φ_{uc} for already known caged NOs are rather poor: 0.02–0.05 for *o*-nitrobenzyl ester caged NOs (CNO-1-4)[15] and 0.05 for $K_2Ru(NO)Cl_5$.[19] The $\varepsilon\Phi_{uc}$ of BNN5Na is $2.7\times10^4\,M^{-1}\,cm^{-1}$ with 300 nm light and $6\times10^3\,M^{-1}\,cm^{-1}$ with 350 nm light; this is much greater than for previously reported CNOs ($\varepsilon\Phi_{uc}$: 15–70 $M^{-1}\,cm^{-1}$ with 360 ± 20 nm light).[15]

The large $\varepsilon\Phi_{uc}$ of BNNs is consistent with photovasorelaxation assay results. The irradiaton of rat aortic tissues loaded with BNN3 or BNN5M with only nM level concentrations for 10 min resulted in NO release sufficient to cause a physiological response in the tissue (Figs. 6 and 7). Further, the photorelaxation response of rat aortic strips was found to attain its maximum with only 3 μM BNN5M loading solution (Fig. 7). Either less of BNN or a weaker uncaging light source may deliver NO at the target tissue, compared to caged NOs having smaller $\varepsilon\Phi_{uc}$.

τ_{uc}

When a caged compound is used to explore very fast biological events, the researcher should use a caged compound having a very small τ_{uc} value. In the stepwise release of two NO molecules from a photoexcited BNN molecule, the first τ_{uc} for the three BNNs is less than 10 ns and the second is 5.6 μs for BNN3 and 5.8 μs for BNN5M in benzene, and 19 μs for BNN5Na in water at 37°C. These τ_{uc1} are several orders of magnitude smaller than that of *o*-nitrobenzyl ester caged compounds, which are usually at μs–ms level.[46,47] Caged NOs having a very small τ_{uc} are useful for kinetically investigating very fast NO events in biological systems, e.g. NO diffusion kinetics in tissue; sudden NO delivery for the time-resolved X-ray crystallographic analysis of NO-requiring enzymes such as soluble guanylate cyclase,[48] cytochrome P-450nor[49] and nitrilehydratase;[50] the determination of second-order rate constants for NO-binding to metalloenzymes and its models, and kinetics of biological NO signaling. This was exemplified by the successful determination of the second-order rate constant for the coordination of NO with $TPPCo^{II}$ and NO by LFP of a solution of $TPPCo^{II}$ and BNN5M (Figs. 1 and 2), i.e. $1.99\pm0.03\times10^9\,M^{-1}\,s^{-1}$ in benzene at 37°C, revealing that BNNs are promising for use in studying fast NO reactions in vivo and in vitro.

An experimental result shown in Fig. 2(A) for LFP of 100 μM BNN5M solution was obtained by using rather a higher concentration than that applied in actual biological chemistry. As mentioned above, 3 μM is maximum concentration in vasorelaxation assay. The second-order rate constant for the combination of the *N*-radical intermediate and NO to reproduce BNN5M proceeds with the second-order rate constant on the order of $10^9\,M^{-1}\,s^{-1}$.[32] However, 3 μM is maximum concentration required in vasorelaxation assay, so the μM level of BNN appears sufficient in biological applications. Under such conditions the bimolecular process of the recombination of the *N*-radical intermediate and NO cannot compete with the unimolecular process of the second NO extrusion reaction of the *N*-radical.

Localization in aortic tissue

A third BNN advantage is that three different BNN solubilities are available. (a) BNN5Na is only soluble in aqueous compartments and is membrane-impermeant (Table 1, entries 3 and 4). If BNN5Na is introduced into an outer cellular aqueous medium, the reagent remains there and does not permeate into the cell. (b) BNN3 is incorporated into vascular smooth muscle cells. Since BNN3 is water-insoluble, it localizes at lipids in cells. (c) BNN5M is also membrane-permeant, but it is only slightly water-soluble and is hydrolysed by cytosolic esterases to BNN5Na, which is membrane-impermeant and hence remains in the cell.

Conclusion

Choosing a suitable BNN and uncaging light source of 300–360 nm, the NO can be delivered at a precise time and space resolutions at a specific compartment in a living system. At least for the contractile apparatus and in hydrolysis experiments of 100 μM BNN5M using cultured smooth muscle cells, we observed no noticeable BNN cytotoxicity.

Experimental

Materials

BNN3 and BNN5 were prepared as described elsewhere.[32] BNN5Na was prepared by treating BNN5 methanol solution with two equimolar amounts of 2 M NaOH aqueous solution. Crystals liberated were collected and dissolved in a minimum volume of water. Pure pale yellow BNNNa crystals were precipitated by adding methanol to the solution. BNN5Na: ^1H NMR (D_2O, 200 MHz, in δ): 7.77 (s, 4H, aromatic), 4.69 (s, 4H, CH_2), UV: λ_{max}, 300 nm (ε: 13,500 M cm^{-1}) in 0.1 M sodium phosphate buffer, pH 7.4. BNN5M was prepared by slowly adding a diazomethane ether solution to a BNN5 methanol solution until yellow diazomethane no longer faded at room temperature. Solvents were evaporated to dryness in a vacuum, leaving a pale yellow solid that, upon alumina chromatography (eluent: dichloromethane) and recrystallization from a mixture of dichloromethane and ethyl acetate yielded pale yellow pure BNN5M crystals. BNN5M mp.144–145°c (decomp.), Anal. calcd. for $C_{12}H_{14}N_4O_6$: C, 46.45; H, 4.52; N, 18.06, found C, 46.36; H, 4.54; N, 17.85, ^1H NMR ($CDCl_3$, 200 MHz, in δ):7.27 (s, 4H, aromatic), 4.71 (s, 4H, CH_2), 3.81 (s, 6H, -OCH_3); IR (nujol, in cm^{-1}): 1738, 1143, 1116, 1520. UV: λ_{max}, 292 nm (ε: 13,700 M cm^{-1}) in methanol.

Methoxamine, fetal bovine serum (FBS), DMEM, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), phosphate buffered saline solution (PBS), fetal bovine serum (FBS), and phenyl-methylsulfonyl fluoride (PMSF) were purchased from Sigma, St. Louis, MO and Cremophore-EL from Aldrich, Milwaukee, WI. Acetylcholine and other special grade reagents were obtained from Wako Pure Chemicals, Osaka, Japan.

Krebs–Ringer's solution consisted of (in mM): NaCl (113); KCl (4.8); $NaHCO_3$ (25); KH_2PO_4 (1.4) $CaCl_2$ (2.2); $MgSO_47H_2O$ (1.4); and glucose (5.5). Depolarizing KCl solution consisted of (in mM): NaCl (63.2); KCl (48); $NaHCO_3$ (25); KH_2PO_4 (1.4) $CaCl_2$ (2.2); $MgSO_47H_2O$ (1.4). Homogenization buffer solution for cultured A10 cells consisted of (in mM): Tris–HCl buffer of pH 7.5 (20); sucrose (250); EGTA (0.5); PMSF (1); 20 mg/mL aprotinin.

Oxyhemoglobin freshly isolated from human blood was a generous gift from Professor Yasuhiko Yamamoto, University of Tsukuba.

Laser flash photolysis (LFP)

A BNN solution in 10×10 mm UV cuvette with a three way cock was irradiated with a 308 nm wavelength laser pulse having a 20 ns width from an excimer laser (Lambda Physik LPX) under argon. Absorbance time courses of transient species in the UV–VIS region formed by laser flash photolysis were measured using a system consisting of a xenon flash lamp (Wacom KXL-151, 150 W), a photomultiplyer (Hamamatsu Photonics R 928), and a storage scope (Iwatsu TS-8123).

Preparation of rat aortic strips and tension readings

Aortic segments were obtained from 12-week-old male Wister rats (400–450 g). After washing inside with Krebs–Ringer solution, segments were cut into 3×13 mm helical strips. The endothelium was denuded by gentle rubbing with small swabs, and the removal was confirmed by loss of a relaxant response to acetylcholine. Arterial strips were placed by means of thread in a jacketed drop-away organ bath containing 20 mL Krebs–Ringer's solution maintained at 37°C and aerated with 95% O_2–5%CO_2. Arterial strips were equilibrated at a passive tension of 0.4 g until the contractile response caused by 50 mM K^+ became a steady state.[51] The upper thread of each strip was connected to a force-displacement transducer (Nihon Kohden TB-611T), while the lower thread was anchored at the bottom. Contractile responses were recorded using a thermal pen recorder (Nihon Kohden WT-647G).

Caged NO assay

All procedures were done in a dark room under red light. BNN5M solution (1 mM) was prepared by adding a 2.33 mg BNN5M solution in 50 μL of dimethyl sulfoxide (DMSO) to 1450 μL of 10% aqueous Cremophor-EL solution followed by gentle ultrasound sonication for 20 s. The solution was diluted with 10% aqueous Cremophor-EL solution so that, when 40 μL of the solution was added to a 20 mL Krebs–Ringer's solution organ bath containing aorta strips, the final BNN5M concentration became the desired value. BNN5M was loaded by adding 40 μL of the BNN5 solution to the organ bath and equilibrated for predetermined time intervals. After washing two times with normal Krebs–Ringer's solution, 10 μM methoxamine was applied to strips, which caused tension development that plateaued within 20 min. Tissue was irradiated with 300 or 360 nm light introduced in the solution through a 0.8 mm quartz optical fiber from a JASCO high-power monochrome light source equipped with a 500 W xenon lamp and a light-path shutter. The light source power was 0.26 mW at 300 nm and 045 mW at 360 nm from the top of the fiber, which was continuously moved by hand so that isostrength light reached all tissue. BNN3 and BNN5Na were assayed in the same way as BNN5M; while a BNN5Na solution was prepared by dissolving BNN5Na in water.

BNN5M Hydrolysis assay in cultured smooth muscle cells

A10 cells were cultured in DMEM with 10% FBS, washed two times with fresh DMEM, and then 10 mL of DMEM and 100 μL of BNN5M/DMSO solution ([BNN5M] = 10 mM) were added to attain a final BNN5M concentration of 100 μM. The mixture was incubated at 37°C. After incubation, cells were rinsed two times with ice-cold PBS until BNN5M was no longer detected in the culture solution, and then lysed in 500 μL of homogenization buffer and homogenized at 4°C. After centrifugation at 150,000 *g* for 30 min at 4°C, the supernatant (cytosolic fraction) was ultrafiltered using a Milipore Ultrafree-MC 30,000 NMWL Filter Unit. The filtrate was freeze-dried to an appropriate volume and subjected to HPLC analysis. The HPLC facility consisted of a Hitachi L 6200 intelligent pump, TOSO ODS 80Ts column (4.6×250 mm), and a Hitachi L4200 UV–VIS detector. The eluent consisted of (volume %) water (70), methanol (30), and phosphoric acid (0.1). In the course of enzymatic hydrolysis of BNN5M, a partially hydrolysed product, monomethyl ester of BNN5, may be present. Since we could not prepare the authentic sample of the half ester, we did not observe measurable peak at longer retention time region of the liquid chromatogram. If the rate determining step of the conversion of BNN5M into BNN5Na in smooth muscle cells is BNN5M hydrolysis to the monoester, the half ester intermediate level is low in cytoplasm.

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