Serial Review

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Guest Editor: Hidehiko Nakagawa

Photocontrollable NO-releasing compounds and their biological applications

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Photocontrollable nitric oxide (NO)-releasing compounds, or caged NOs, are very useful to expose cells or tissues to NO in a spatiotemporally well-controlled manner, e.g., for precise investigations of biological responses to NO and NO-related signaling pathways. We have developed photocontrollable NO releasers based on two mechanisms: photoinduced isomerization reaction of a dimethylnitrobenzene moiety conjugated with a pi-electron system, and photoinduced electron transfer of a moderately electron-rich *N*-nitroso aminophenol moiety linked with an antenna dye moiety. In this review, we describe the development of our photoinduced NO releasers based on these mechanisms, and present examples of cellular and *ex vivo* applications.

***Key Words*: nitric oxide, caged compound, photochemical reaction, vasodilation**

Nitric oxide (NO) is a gaseous cellular signal transduction mediator related to reactive oxygen species (ROS) and oxidative stress signaling.[1] However, because it is a gas under ambient conditions, it is difficult to use directly for biological experiments, especially at low concentrations. NO-releasing compounds (NO donors) are indispensable for such experiments, and indeed, many NO releasers have been investigated and developed. Among them, photocontrollable NO releasers (so-called caged NO) are the most versatile, because their NO release can be spatiotemporally and precisely controlled by light irradiation from outside the experimental system. Such controlled release may mimic physiological NO production, and also may have potential therapeutic applications. Various photocontrollable NO releasers have been developed, and typical examples are shown in Fig. 1.[2–5] We have focused on photocontrollable NO releasers based on two mechanisms, i.e., photoinduced isomerization reaction of a dimethylnitrobenzene moiety conjugated with a pi-electron system, and photoinduced electron transfer of a moderately electron-rich *N*-nitroso aminophenol moiety linked with an antenna dye moiety. We have also demonstrated their suitability for biological applications. In this review, I focus on recent advances in our laboratory on photocontrollable NO releasers and their applications.

NO Release via Photoisomerization of a Nitrobenzene Moiety

During their investigation of the carcinogenicity of benzo[a]pyrene, Fukuhara and Miyata found that 6-nitrobenzo[a]pyrene releases NO upon photoirradiation, apparently via photoinduced isomerization of the nitro group to aryl nitrite ester, followed by homolytic dissociation of the N-O bond. The nitro group in benzo[a]pyrene is considered to be in a twisted conformation with respect to the aromatic ring due to steric hindrance with H atoms at the peri-position. Although intense short-wavelength UV irradiation can induce isomerization of simple nitrobenzenes, we expected that a twisted conformation would facilitate the isomerization reaction. Based on this consideration, our group developed compounds bearing a 2,6-dimethylnitrobenzene (DNB) moiety, in which the nitro group is assumed to be in the twisted conformation due to the presence of the two vicinal methyl groups. To confirm the validity of this strategy, we synthesized DNB-type 3,5-dimethyl-4-nitrostilbene derivatives. Indeed, we found that upon photoirradiation in the UVA range (325–385 nm), they released NO both *in vitro* and in cells.[6] As a cell-applicable DNB-type NO releaser, we then developed Flu-DNB (Fig. 2), which could be loaded into the cell membrane, where it released NO intracellularly upon UVA irradiation (Fig. 3).[7,8] Flu-DNB is also available for *in vivo* experiments. Under anesthesia, a small hole was made in the skull and dura of a mouse, and the mouse brain was treated with Flu-DNB in artificial celebrospinal fluid (ACSF). By means of two-photon fluorescence microscopy, we observed that irradiation of vessel walls with a near-infrared (735 nm) pulse laser in brain regions where Flu-DNB was distributed resulted in an increase of the vessel diameter only within the irradiated area.[8] No response was observed after treatment with fluorescein alone (lacking the DNB moiety). These results suggest that Flu-DNB released NO *in vivo* and induced physiological responses in response to irradiation.

We investigated the relationship between the electronic structure and NO-release properties of the DNB-type NO releasers. For this purpose, we developed two DNB-type compounds conjugated with a coumarin chromophore. One was Bhc-DNB, in which the DNB group is located at an unconjugated (cross-conjugated) position with respect to the intramolecular charge transfer (ICT) dipole moment (Fig. 2), and the other was DEAMC-DNB in which the DNB group is conjugated with the ICT dipole moment. When the compounds were irradiated in the absorption band in aqueous solution, we found that Bhc-DNB released NO, while DEAMC-DNB did not. We assumed that direct conjugation of pi-electrons of the chromophore to the DNB moiety would inhibit the isomerization reaction to aryl nitrite due to the increase of

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#He received "SFRR Japan Award of Scientific Excellent" in 2014 in recognition of his outstanding work.

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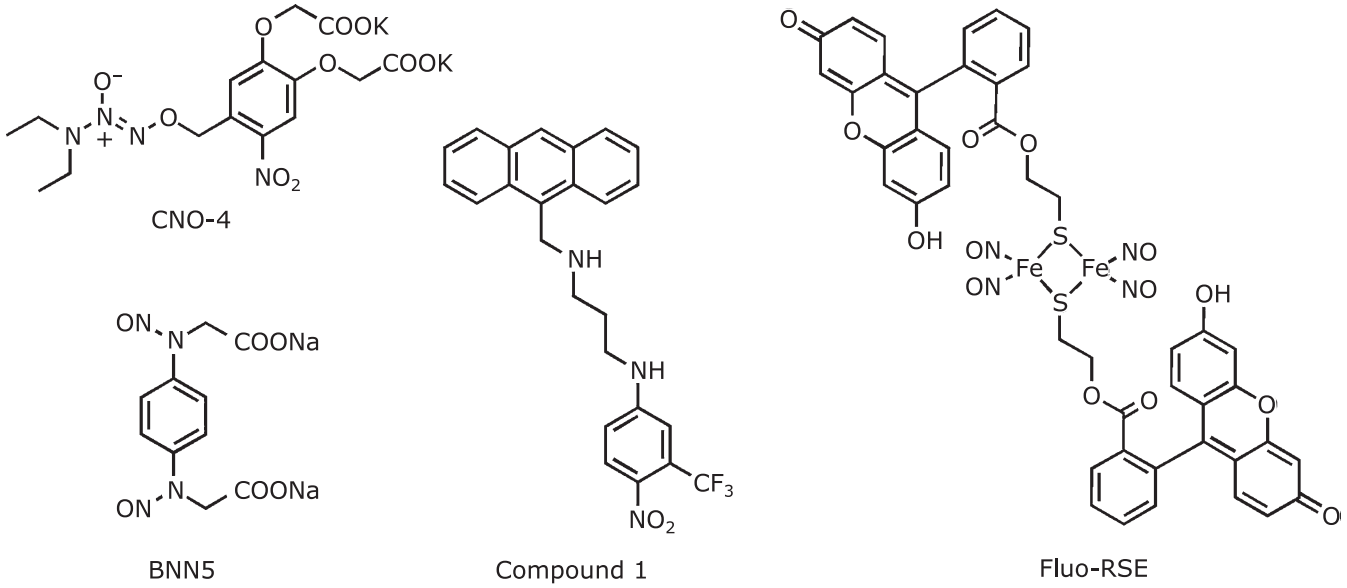

Fig. 1. Examples of known photocontrollable NO releasers. CNO-4,[2] BNN5,[3] compound 1,[4] and Fluo-RSE.[5]

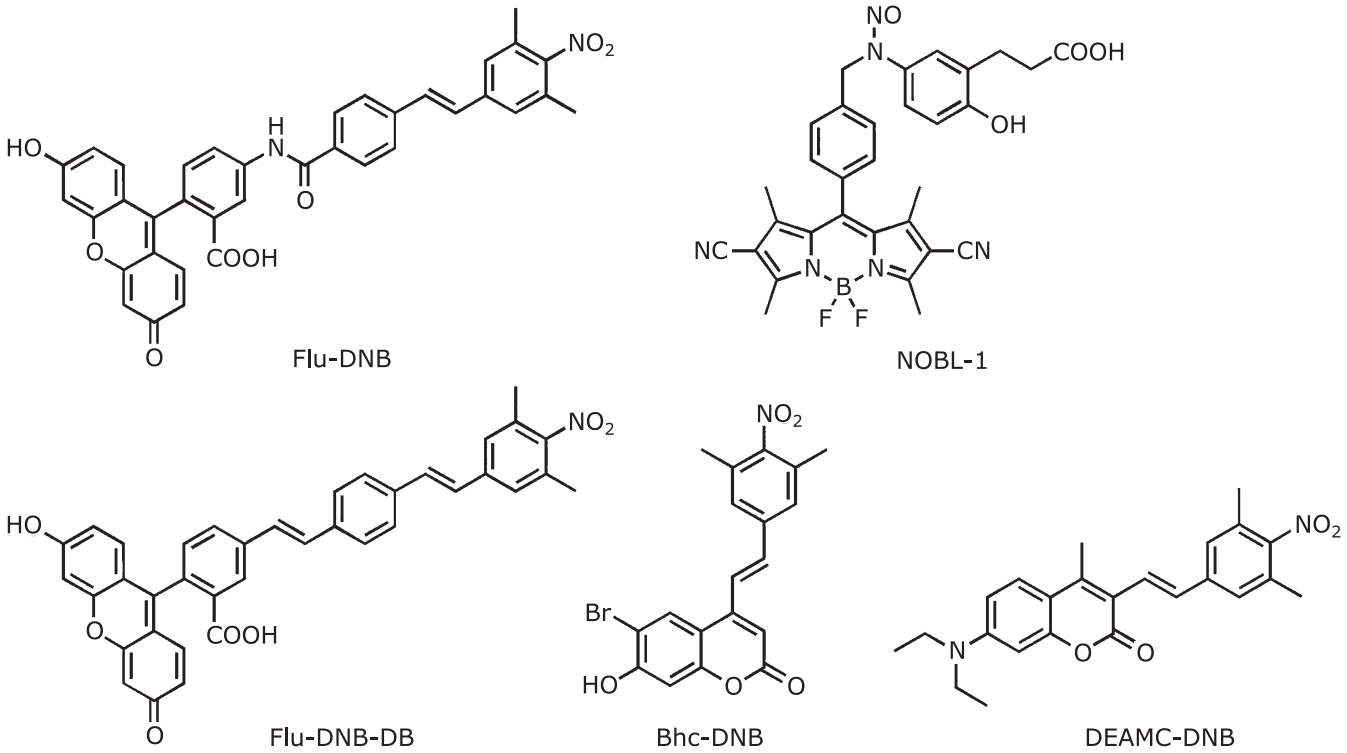

Fig. 2. Our photocontrollable NO releasers. Photocontrollable NO releasers, Flu-DNB, Bhc-DNB, Flu-DNB-DB, and NOBL-1, and a related coumarin derivative DEAMC-DNB.

pi-conjugation between the aryl group and nitro group, whereas the cross-conjugation system would not affect the C-N bond strength, and non-radiative relaxation of the photo-excited molecules might provide energy for isomerization (or reduction of the electron density at the ipso-position of the nitro group in the excited state might facilitate it).[9] Although further investigations will be needed to elucidate the precise mechanisms of DNB isomerization and NO release, it is noteworthy that the nature of the conjugation system affected the NO release efficiency of DNB-type compounds.

We also made use of the above findings to improve Flu-DNB. In Flu-DNB, photoabsorption and NO release are considered to depend on the stilbene moiety. In order to extend conjugation in the molecule, we replaced the amide group connecting the stilbene

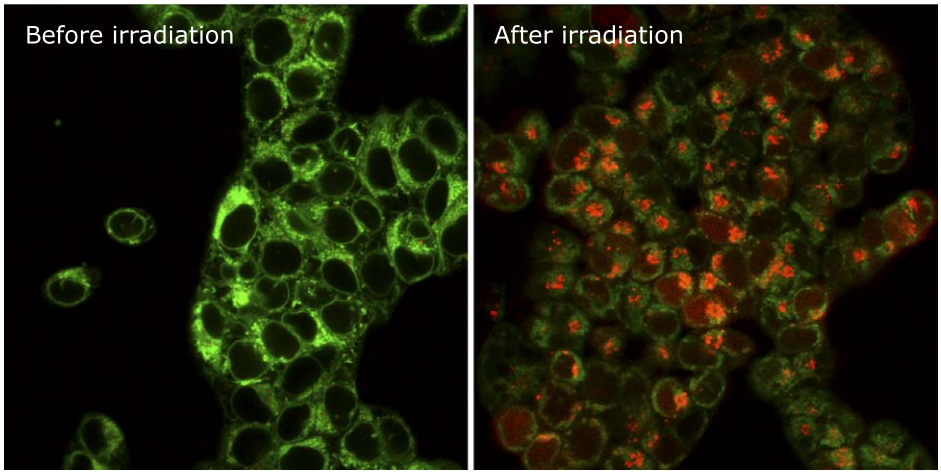

Fig. 3. Intracellular NO release from Flu-DNB in response to UVA irradiation. HCT116 cells were treated with Flu-DNB for 24 h, and then the cells were loaded with DAR 4M AM, a red fluorogenic NO probe. After washing, the cells were irradiated with UVA for 5 min. Increase of red fluorescence indicates release of NO in the cells. Left panel: before irradiation, right panel: after irradiation.

moiety to the fluorescein moiety with a simple olefin linker, which should extend the conjugation to the benzene ring of the fluorescein moiety. We synthesized and evaluated the new DNB-type compound, Flu-DNB-DB (Fig. 2), and found the absorption maximum was shifted to 359 nm, whereas that of Flu-DNB was 322 nm, as expected.[10] We also found that Flu-DNB-DB released NO, though only weakly, upon photoirradiation at 450–480 nm. Although Flu-DNB has absorption in the same range, the longer conjugation in Flu-DNB-DB may enhance the absorption at this wavelength range and facilitate NO release. The two-photon decomposition cross section (δu value) of Flu-DNB-DB was found to be about 8 times higher than that of Flu-DNB [δu (720 nm): Flu-DNB-DB, 0.98; Flu-DNB, 0.12]. We next tried to use Flu-DNB-DB under two-photon conditions. After loading Flu-DNB-DB into HCT116 cancer cells, a small area of a cell was irradiated with a femtosecond-pulse laser at 950 nm in a two-photon microscope system. Within the irradiated cells, a fluorescence increase was observed only at the point of irradiation, meaning that Flu-DNB-DB is available for very fine spatiotemporal control of intracellular NO release (Fig. 4).[10]

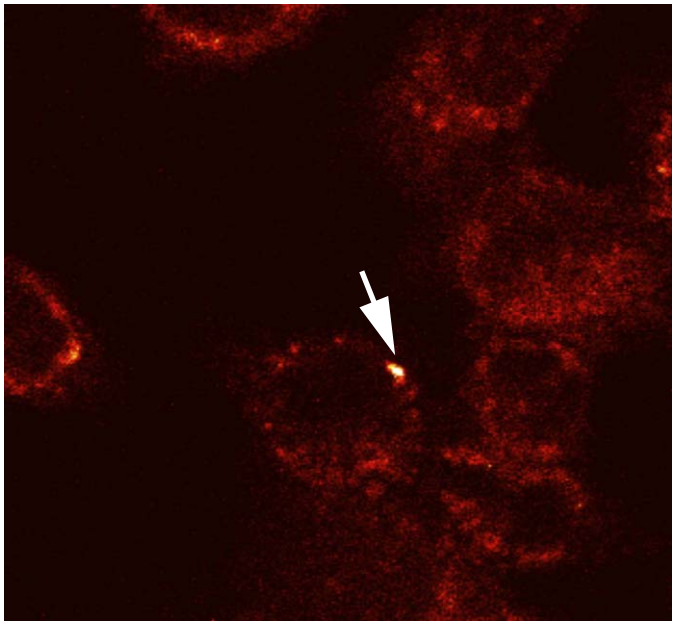

Fig. 4. Highly precise spatial control of NO release from Flu-DNB-DB within a cell in response to femtosecond-pulse laser irradiation at 950 nm. HCT116 cells were treated with Flu-DNB-DB and DAR 4M AM, a red fluorogenic NO probe, and then irradiated with a femtosecond-pulse laser at 950 nm for 0.5 s at the area indicated by the arrow. The photograph shows the confocal image of the cells after irradiation.

Photoinduced Electron Transfer as an NO Releasing Mechanism

We have also investigated another mechanism of NO release in response to photoirradiation. Previously known photocontrollable NO releasers, or caged NO compounds, BNN derivatives (Fig. 1), release two NO molecules upon UV irradiation. The first one is released due to homolytic cleavage of the *N*-NO bond by photoirradiation, while the second one is released via conversion of the unstable radical cation intermediate to more stable quinoneimine form. We hypothesized that an unstable radical cation intermediate like the BNN intermediate might be formed via photoinduced electron transfer reaction through photo excitation of an adjacent visible chromophore. We designed and synthesized an *N*-nitrosoaminophenol derivative conjugated with a BODIPY chromophore, NOBL-1 (Fig. 2). When we irradiated an aqueous solution of NOBL-1 with blue light, where NOBL-1 has an absorption band, in the presence of an ESR spin-trapping reagent, Fe-MGD, or a fluorescent NO probe, DAR 4M, we found that NOBL-1 released NO in response to the photoirradiation, as confirmed by ESR spectroscopy or fluorescence photometry, respectively. After loading of NOBL-1 concomitantly with DAR 4M AM into HEK293 cells, we illuminated cells in a specific area with a 488 nm laser under a fluorescence microscope. Only cells within the illuminated area showed a fluorescence increase, indicating intracellular NO release. This means that NOBL-1 in the cells released NO in response to the visible light irradiation (Fig. 5).[11] We also employed NOBL-1 in an *ex vivo* vasodilation experiment using rat aorta. A strip of rat aorta was placed in a Magnus apparatus with Krebs buffer, and then endogenous NO production was blocked with an NOS inhibitor. The aorta strip was tensioned by adding noradrenaline. After equilibration of tension, the strip was treated with NOBL-1 and irradiated at 470–500 nm. During the irradiation, it was observed that the tension decreased depending on the irradiation time (Fig. 6). No relax-

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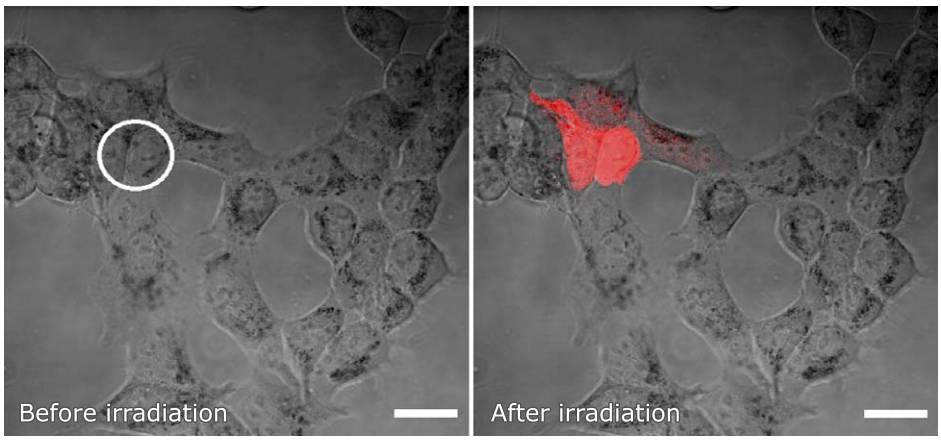

Fig. 5. Spatial control of NO release from NOBL-1 in response to 488 nm laser irradiation. HEK293 cells were treated with NOBL-1 and DAR 4M AM for 1 h. After washing, the cells were irradiated with a 488 nm laser within the area indicated by the circle. Increase of red fluorescence indicates NO release in the cells. Left panel: before irradiation, right panel: after irradiation.

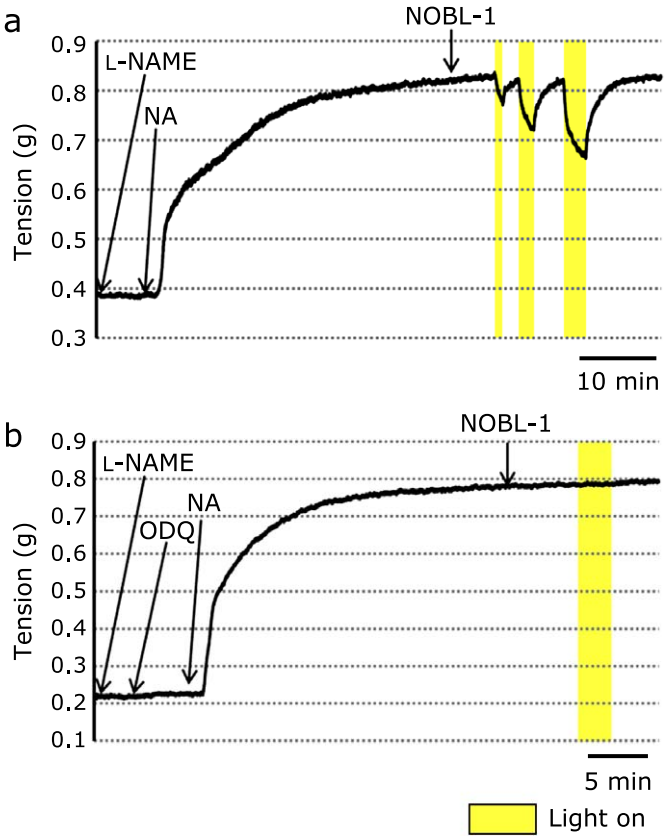

Fig. 6. Vasodilation in a rat aorta strip in response to photoinduced NO release from NOBL-1. Rat aorta was treated with L-NAME (10 μM) and noradrenaline, followed by NOBL-1 in Magnus tubes. (a) Each tube was then irradiated (470–500 nm) for 1, 2 or 3 min (as indicated by the yellow shading). (b) ODQ (10 μM) was added before addition of NOBL-1. Light irradiation was conducted for 3 min. Adapted with permission from *J Am Chem Soc*, **136**, 7085–7091 (2014). Copyright (2015) American Chemical Society.

ation was observed upon irradiation in the presence of ODQ, a soluble guanylyl cyclase inhibitor. These results indicate that NO released from NOB-1 by photoirradiation successfully induced vasodilation in rat aorta tissue via the physiological NO-dependent signaling pathway.

In conclusion, we have developed photocontrollable NO releasers using two different types of photoinduced NO releasing reaction, which are responsive to visible or longer-wavelength light. Firstly, by connecting a nitrobenzene moiety to various pi-conjugation systems, we have developed a series of DNB-type photocontrollable NO releasers, in which photo-dependent isomerization of arylnitro group is the key reaction. In the second type of photocontrollable NO releaser, exemplified by NOBL-1, the key mechanism inducing homolytic dissociation of the *N*-nitroso bond is intramolecular photoinduced electron transfer. Flu-DNB and Flu-DNB-DB release NO upon irradiation with UVA-visible light, as well as upon femtosecond-pulse laser irradiation in the near-infrared range. These DNB-type NO releasers are applicable to cultured cells and living mouse brain. NOBL-1 releases NO upon irradiation with blue (470–500 nm) light, and is applicable to cells and tissues.

These NO releasers are useful tools to investigate biological responses to NO, especially diffusion and time-dependent effects, because they enable precise spatiotemporal control of NO release simply by using photoirradiation. They might also be useful to investigate and develop new phototherapies based on the pharmacological action of NO.

Abbreviations

DNB	dimethylnitrobenzene
NO	nitric oxide

Conflict of Interest

No potential conflict of interest were disclosed.

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