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Original Contribution

A novel fluorescent probe for the detection of nitric oxide in vitro and in vivo

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ABSTRACT

Fluorescence imaging of nitric oxide (NO) in vitro and in vivo is essential to developing our understanding of the role of nitric oxide in biology and medicine. Current probes such as diaminofluorescein depend on reactions with oxidized NO products, but not with nitric oxide directly, and this limits their applicability. Here we report the formation of an imaging probe for nitric oxide by coordinating the highly fluorescent chemical 4-methoxy-2-(1*H*-naphtho[2,3-*d*]imidazol-2-yl)phenol (MNIP) with Cu(II). The coordination compound MNIP–Cu reacts rapidly and specifically with nitric oxide to generate a product with blue fluorescence that can be used in vitro and in vivo. In the present study MNIP–Cu was used to reveal nitric oxide produced by inducible nitric oxide synthase in lipopolysaccharide (LPS)-activated macrophages (Raw 264.7 cells) and by endothelial nitric oxide synthase in endothelial cells (HUVEC). MNIP–Cu was also used to evaluate the distribution of nitric oxide synthesis in a model of acute liver injury induced by LPS and D-galactosamine in mice. The results demonstrate that MNIP–Cu can act as a novel fluorescent probe for nitric oxide and has many potential applications in biomedical research.

Introduction

Nitric oxide (NO) is a multifunctional free radical involved in cell signaling and cellular physiology [1,2]. However, a significant limitation of current research methods is the ability to visualize the spatial and quantitative formation of NO in vivo and in vitro. There are well-developed, small-molecule-based fluorescent probes for NO, including *o*-diaminofluoresceins (DAFs), diaminorhodamines, and *o*-diaminocyanines [3–5]. Currently DAFs are frequently used as NO fluorescent probes. However, the fluorescent changes in DAFs require reaction with oxidized NO products, not NO itself, which limits their applicability [6]. Thus, DAFs cannot be used to image NO formation under hypoxic or anoxic conditions, such as in tumors, in which oxidation of NO is decreased.

Lim et al. have demonstrated that several novel copper-coordinated fluorescent complexes [6,7] react directly with NO. Copper-coordinated fluorescent complexes exhibit many advantages over current NO probes, not least because they react with NO directly and do not require the presence and detection of an oxidized product.

Derivatives of 2-(1*H*-naphtho[2,3-*d*]imidazol-2-yl)phenol are a series of fluorescent compounds that exhibit intense emission properties via an excited-state intramolecular proton transfer [8,9]. These derivatives have yielded a new series of fluorescent probes [10,11] or laser dyes [12,13], which can coordinate with cupric ions to form stable nonfluorescent copper coordination compounds [14,15]. In this study we have shown that some of these compounds react with NO and can be used for fluorescent imaging of NO in vitro and in vivo.

In this study we report the synthesis of 4-methoxy-2-(1*H*-naphtho[2,3-*d*]imidazol-2-yl)phenol (MNIP), which coordinates with Cu(II) to form a stable coordination compound, MNIP–Cu. MNIP–Cu reacts rapidly and specifically with NO to generate a blue fluorescence. Matrix-assisted laser desorption/ionization, time-of-flight mass spectrum (MALDI-TOF), ESR, HPLC, and FT-IR spectra were used to identify the mechanism of copper reduction and fluorophore nitrosation. Importantly the probe is cell-permeative and can be used to image cellular NO with high resolution. To determine whether this probe could be used in vivo, we used normal mice and a model of severe liver injury induced by galactosamine and lipopolysaccharide (LPS) [16]. Injection of MNIP–Cu into a peripheral or the portal vein, followed by fluorescence detection in liver cryosections, enabled the quantitative measurement of hepatic NO synthesis in normal mice and mice with severe liver injury in vivo.

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Materials and methods

Synthesis of MNIP and MNIP–Cu

MNIP was synthesized according to previously published methods [17]. To form MNIP–Cu, MNIP was first dissolved in dimethyl sulfoxide (DMSO) to form a 1 mM solution. Then 20 μl of 50 mM copper sulfate aqua solution was added to 1 ml MNIP solution to the final MNIP: copper ratio of 1:1. The mixture was stirred at room temperature for 5 min to form a stable yellow MNIP–Cu solution.

Characterization of MNIP and MNIP–Cu

MNIP was characterized by ^{1}H NMR, ^{13}C NMR (DRX-500, Bruker, Switzerland), EI-MS (JMS-700, JOEL, Japan), and elemental analysis (JSX-3202M, JOEL, Japan). MNIP–Cu was characterized by MALDI-TOF (Ultraflex II, Bruker, Switzerland), ESR (FE1X, JOEL, Japan), UV–Vis (UV 3000, Shimadzu, Japan), and FT-IR (Nicolet Nexus 870, Thermal Fisher Scientific, USA). NMR spectra were recorded in d6-DMSO with tetramethylsilane (TMS) serving as the internal standard.

Fluorescence assay of MNIP–Cu with NO

The fluorescence measurements were carried out on a Hitachi Model F-4500 spectrophotometer (Hitachi, Tokyo, Japan). MNIP–Cu was dissolved in water/DMSO (v/v 1/1). NO and its standard solutions were prepared according to the method of Huang et al. [18]. In brief, NO was generated by slowly dropping 2 M H_2SO_4 into saturated $NaNO_2$ solution. The gas was forced through a 30% NaOH solution and water to trap NO_2. Before the addition of H_2SO_4, the entire apparatus was degassed carefully with nitrogen for 30 min. To produce a saturated NO solution (at 20°C, 1.8 mM), 10 ml deoxygenated distilled water was bubbled with NO for 30 min and kept under NO atmosphere until use. Standard solutions were freshly prepared for each experiment and kept in a glass flask with a tight rubber seal. The testing concentration of MNIP–Cu was 1 μM. The fluorescence curves were recorded at 0, 0.5, and 5 min after the reaction with 1000 eq of NO.

Specificity and sensitivity assay of MNIP–Cu with NO

The specificity of 1 μM MNIP–Cu to NO over other reactive species in biological systems was determined by the addition of 100 eq of H_2O_2, NO_2^-, NO_3^-, NH_4^+, $ONOO^-$, and NO (g) for 1 h, and fluorescence intensities of MNIP–Cu at 492 nm were recorded.

The sensitivity of MNIP–Cu to NO was also tested in Raw 264.7 cell lysate and Balb/c mouse plasma using a NO-releasing agent, *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP; Sigma–Aldrich). SNAP at a concentration of 100 μM can produce 1.4 μM NO per minute in PBS with pH 7.4 at 37°C [19]. The test concentration of MNIP–Cu was 10 μM. $F_{492\ nm}$ was recorded. Fluorescence intensities were normalized with respect to the emission of MNIP–Cu.

HPLC analysis of the reaction of MNIP-Cu with NO

The HPLC column used was C18, 4.6×250 mm, 5 μm, 300 Å. The mobile phase, acetonitrile (60% v/v), 0.01 M ammonium acetate, and 0.5% acetic acid (40%), was pumped with a flow rate of 500 μl/min. The injection volume for each sample was 20 μl. The detection system was Shimadzu SPD-10A (Shimadzu, Japan) with a UV detection wavelength of 360 nm and a sample concentration of 250 μM. To confirm the elution time of MNIP–NO, 250 μM MNIP–Cu and NO were reacted and analyzed.

To confirm that the application of MNIP–Cu in LPS-activated Raw 264.7 cells generated the fluorescent MNIP–NO, we stimulated Raw 264.7 cells with 500 ng/ml LPS. MNIP–Cu was added simultaneously, to a final concentration of 50 μM. Cells (5×10^6) were digested and lysed in 5 ml PBS after 12 h. One milliliter of chloroform was added to the cell lysate to extract the fluorescent products. A solution of 50 μM MNIP–Cu was also extracted with chloroform to serve as a nonreacting control. After the chloroform was vaporized, the samples were resolved in 100 μl acetonitrile before analysis.

Fluorescent detection of NO in macrophages and endothelial cells

Raw 264.7 macrophages were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum to a confluence of 85%. Cells were then stimulated with 500 ng/ml LPS, while 10 μM MNIP-Cu was applied simultaneously. The fluorescence alteration was monitored at 2-h intervals by fluorescence microscopy (TE2000-U, Nikon, Japan), using the 330–385 nm bandpass filter and emission filter BA420. MNIP–Cu was also incubated with unstimulated cells to evaluate its stability and fluorescence background.

To confirm that the cellular fluorescence was associated only with NO production, we used the following reagents: an inducible nitric oxide synthase (iNOS) inhibitor, *N*-[3-(aminomethyl)benzyl] acetamidine, dihydrochloride (1400w) [20]; an NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (PTIO); butylated hydroxytoluene (BHT); superoxide dismutase (SOD); and catalase. For 1400w, 500 ng/ml LPS was added 4 h after 100 μM 1400w treatment, followed by a 10 μM MNIP–Cu application. For PTIO, after LPS addition, 500 μM PTIO was added at the set time (3, 5, 7, 9, 11, 13, or 19 h after LPS application), and 10 μM MNIP–Cu was added 0.5 h later. BHT, SOD, and catalase were added simultaneous with LPS (BHT, 100 μM; SOD and catalase, 100 U/ml), followed by an application of 10 μM MNIP–Cu. Fluorescence images were recorded at 4, 6, 8, 10, 12, 14, and 20 h after LPS addition.

Images from various time checkpoints were analyzed using ImageJ software (U.S. National Institutes of Health; version 1.38x). Fluorescence intensity per cell area at each time point was calculated using a minimum of five images.

Meanwhile, human umbilical vein endothelial cells (HUVEC) were incubated with DMEM supplemented with 10 μM calcium ionophore A23187 (Sigma–Aldrich) or 100 nM 17-β-estradiol (Sigma–Aldrich) [21,22]. MNIP–Cu (10 μM) was applied immediately. The fluorescence alteration was monitored and recorded at 1-h intervals. Images of untreated HUVEC with MNIP–Cu were also taken. To confirm further that the fluorescence was derived from the reaction with nitric oxide, a potent endothelial NOS (eNOS) inhibitor, *N*5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO), was applied at a concentration of 500 μM, 4 h before the calcium ionophore or 17-β-estradiol application [23]. MNIP–Cu (10 μM) was added to L-NIO-treated HUVEC to generate negative control images.

To test the cytotoxicity of MNIP–Cu and its reaction product with NO, we performed a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay using Raw 264.7 cells and HUVEC [24].

In vivo fluorescence detection in mice

Male Balb/c mice (6–8 weeks, 18–20 g) were obtained from the Model Animal Center of Nanjing University (Nanjing, China). The study conducted in this report complied with the current ethical regulations of the animal research committee of the university. Forty-five mice were randomly divided into two groups. One group of 15 mice was set as the control group, whereas the other 30 mice were set as the test group. Normal saline (0.2 ml) was injected intraperitoneally in the control group. LPS (5 μg/kg) together with D-galactosamine (GalN) (300 mg/kg) in normal saline was injected