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Real time electrochemical investigation of the release, distribution and modulation of nitric oxide in the intestine of individual zebrafish embryos

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ABSTRACT

Nitric oxide (NO) is an important signaling molecule that has been implicated in a variety of physiological and pathophysiological processes in living organisms. NO plays an important role in embryonic development in vertebrates and has been reported to influence early organ development and plasticity. Quantifying NO in single embryos and their developing organs is challenging because of the small size of the embryos, the low dynamically changing concentration and the short life-time of NO. Here, we measured the distribution of NO in the intestine of live zebrafish (*Danio rerio*) embryos in physiological conditions and under the influence of therapeutic agents. NO measurements were performed using a miniaturized electrochemical sensor fabricated on a single carbon fiber (CF) which enables quantitative real time *in vivo* monitoring, and by fluorescence imaging using the 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA) dye. NO production was detected in the middle segment the intestine at a level of 3.78 (± 0.64) μM, and at lower levels in the anterior and posterior segments, 1.08 (± 0.22) and 1.00 (± 0.41) μM respectively. In the presence of resveratrol and rosuvastatin, the intestinal NO concentration decreased by 87% and 84%, demonstrating a downregulating effect. These results indicate the presence of variable micromolar concentrations of NO along the intestine of zebrafish embryos and demonstrate the usefulness of CF microelectrodes to measure quantitatively the NO release at the level of a single organ in individual zebrafish embryos. This work provides a unique approach to study in real time the modulatory role of NO *in vivo* and contributes to further understanding of the molecular basis of embryonic development for developmental biology and drug screening applications.

1. Introduction

Nitric oxide (NO) is an important signaling molecule that is involved in a wide variety of physiological and pathophysiological processes in living organisms. Since its discovery as a smooth muscle relaxant factor [1], NO has been linked to many other functions, including neurotransmission [2], immune and inflammatory responses, modulation of ion channels, and cardiac regulation [3]. NO is produced by nitric oxide synthase (NOS) enzymes from L-arginine substrate. Regulation of NO formation in tissue and organs by targeting the functions of the NOS enzymes is a potential therapeutic approach for treatment of diseases [4], including cardiovascular disease [5,6], cancer [7], and diabetes [8]. Although much is known about the different roles of NO as a signaling molecule, the molecular mechanisms involved in NO production and regulation are very complex and difficult to study in complex biological environments. Once produced, NO can have both beneficial and damaging effects [2], depending on the site and rate of production, concentration and redox environment. NO is highly reactive with a lifetime of less than 1 s and it can rapidly diffuse and react with oxygen, oxygen radicals, or proteins to more stable products [9]. Potential modulation and measurement of the highly dynamic process of NO signaling in biological systems is challenging because of the limited analytical techniques that can enable direct detection with the required sensitivity and spatial resolution.

Zebrafish (*Danio rerio*) share more than 70% of their genes with humans, making them an excellent model for studying organ development, origin and mechanism of diseases, as well as inflammation and toxicity [10]. Zebrafish embryos develop rapidly and are transparent, which provide additional advantages for visual inspection and easy localization of organs. Zebrafish are an emerging model for drug discovery, enabling rapid high throughput screening of their therapeutic efficacy at significantly lower costs than with other vertebrate models. There is increasing evidence that zebrafish are a useful model to study intestinal inflammation and injury [11]. However, little is known about the presence and distribution of NO in the intestine of zebrafish embryos, which is essential for understanding signaling functions and

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regulation. Traditionally, production of NO in developing zebrafish has been detected using fluorescence spectroscopy with NO-specific dyes [12], but achieving quantitative analysis *in vivo* is difficult because the fluorescent intensity can be affected by the presence of other redox species and endogenous auto-fluorescence from the zebrafish tissue [13,14]. High performance chromatography has been reported as an alternative [15]. Our lab has pioneered a method to implant a microelectrode in the intestine of live zebrafish embryos to quantitatively measure *in vivo* levels of intestinal neurotransmitters using DPV [16–18]. Amatore et al. have used miniaturized carbon fiber (CF) microelectrodes modified with platinum black to determine the complete flux of reactive oxygen and nitrogen species, including NO, released by single cells and immunologically activated macrophages [19–23] and study the antioxidant activity of chemical compounds with potential therapeutic applications using relevant cell lines [24,25]. Miniaturized CF microelectrodes enable real time measurements with high spatial resolution, which is needed to quantify biomarkers at the production site in the organs in individual embryos.

Herein, we expand the use of this technique to measure *in vivo* levels of intestinal NO and quantify the change in the NO concentration due to exposure to pharmacological agents targeting the NOS system. For that, we fabricated a miniaturized CF microelectrode modified with a selected NO-specific catalytic material, nickel (II) phthalocyanine-tetrasulfonic acid tetrasodium salt (NiTSPc). Using this technology, we were able to determine the spatial distribution of NO along the intestine of 5 days post-fertilization (dpf) zebrafish embryos. Measurements were performed with the electrode inserted at different segments within the intestine of wild type embryos and in embryos with pharmacologically altered NOS activity. Electrochemical measurements were compared side-by-side with fluorescence imaging with the 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA) dye that allows visualization of NO in live vertebrates [18] by forming a fluorescent DAF-triazole derivative upon reaction with NO [26–28]. These measurements revealed variable low micromolar concentrations of NO along the intestine of embryos in physiological conditions, which may relate to the anterograde and retrograde intestinal contractions. Furthermore, we have studied the regulating effect on NO in the case of two therapeutic drugs (resveratrol and rosuvastatin). It was observed that both drugs have a downregulating action, as intestinal NO levels have been altered significantly after exposure. Overall, the use of a miniaturized electrochemical sensor provides an approach to study the modulatory role of NO *in vivo* and contributes to further understanding of the molecular basis of intestinal development for developmental biology and drug screening applications.

2. Materials and methods

2.1. Electrode materials and reagents

Carbon fibers (~5 μm diameter) were obtained from World Precision Instruments. Silver conductive epoxy was purchased from MG Chemicals. 5-minute non-conductive epoxy was obtained from Devcon. 2-allylphenol, nickel (II) phthalocyanine-tetrasulfonic acid tetrasodium salt, Nafion (5% mixed in aliphatic alcohol), hemoglobin bovine, sodium hydrosulfite, L-ascorbic acid sodium salt, dopamine hydrochloride, norepinephrine hydrochloride, agar, and rosuvastatin calcium were purchased from Sigma-Aldrich. Allylamine, 2-butoxyethanol, and calcium chloride were obtained from Acros Organics. Methanol and dimethylsulfoxide were purchased from J. T. Baker. 2-propanol, sodium hydroxide, sulfuric acid, hydrogen peroxide, sodium chloride, and magnesium sulfate were purchased from Fisher Scientific. Sodium phosphate dibasic was purchased from Spectrum. Potassium phosphate monobasic and potassium chloride were purchased from LabChem, Inc. Sodium nitrite and serotonin hydrochloride were purchased from Alfa Aesar. Epinephrine was obtained from MP Biomedicals. L-NAME hydrochloride was bought from Tocris Biosciences. Spermine NONOate was purchased from Cayman Chemical. 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA) was purchased from Life Technologies. Resveratrol was purchased from TCI America. E3 medium (pH 6.9–7.2) contains 5 mM sodium chloride, 0.17 mM potassium chloride, 0.33 mM magnesium sulfate, and 0.33 mM calcium chloride in deionized water. All solutions were prepared with purified water (18 MΩ, Millipore, Direct-Q System).

2.2. Instrumentation

Electrochemical measurements were performed with a CH1030a electrochemical analyzer (CH Instruments Inc.). All experiments were carried out with a three-electrode electrochemical cell equipped with a Ag/AgCl/3M NaCl reference electrode (CH111, CH Instruments Inc.) and a platinum wire counter electrode. The working electrode was a lab-made modified carbon fiber microelectrode fabricated from a single carbon fiber. A diamond particle whetstone microgrinder (Model EG-4, Narishige, Tokyo, Japan) was used for polishing of microelectrodes. An optical microscope (Nikon SMZ1000 Stereomicroscope) was used for preparation of microelectrodes and zebrafish embryos manipulation. Visualization of fluorescent samples was accomplished using an inverted Fluorescent Zeiss Axio Observer A1 with the μManager software. Images were processed and analyzed using Image J software.

2.3. Fabrication of carbon fiber microelectrodes

Carbon fiber microelectrodes were prepared according to our previously reported procedure [18]. The fiber at the tip of the electrode was cut ~0.5 mm short using a scalpel. The protruding fiber was insulated electrochemically using a mixture of 0.4 M allylamine, 0.23 M 2-allylphenol, and 0.23 M 2-butoxyethanol in water/methanol (1:1, v/v), according to a reported protocol [19]. The tip of the microelectrode was polished at an angle of 45° for ~5 min using a whetstone microgrinder. The freshly exposed carbon surface was cleaned by dipping in isopropanol for 1 min and by repeated fast-scan cyclic voltammetry between −0.4 and 1.4 V, 500 V/s, in 0.1 M PBS (pH 7.5), until a consistent voltammogram was obtained. The electrodeposition of NiTSPc was done from a N_2-purged solution of 2 mM NiTSPc in 0.1 M NaOH, by applying a constant potential of 1.2 V until the electrodeposition current reached 4 nA. Nafion modification was achieved by dipping the electrode in a 5% (v:v) solution for 5 s, followed by curing at 80 °C for 10 min.

2.4. NO preparation

NO stock solution was prepared based on a procedure reported elsewhere [18,29]. The NO was collected in 0.1 M PBS (pH 7.5). The concentration of the stock was determined before each experiment using a UV–Vis assay based on the reaction between NO and oxyhemoglobin [30].

2.5. Characterization of the microelectrode

After preparation, the NiTSPc-modified microelectrodes were conditioned by differential pulse voltammetry (DPV) between 0.4 and 1.2 V, using a scan increment of 0.004 V, for 5 cycles. The pulse amplitude, width, and period were 50 mV, 50 ms, and 500 ms respectively. A background that was obtained in the absence of NO was subtracted from each voltammogram produced in the presence of NO. Same parameters were used for all *in vitro* and *in vivo* measurements. Characterization of the microelectrode was done in 0.1 M PBS (pH 7.5), including calibration curve and interference study. A second calibration curve was built in E3 medium to be used for *in vivo* measurements.