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Monitoring of in vivo manipulation of nitric oxide synthases at the rat retina using the push–pull perfusion sampling and capillary electrophoresis

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ABSTRACT

Proteins play a variety of functional roles in tissues that underlie tissue health. The measurement of protein function is important to both understand normal and dysfunctional tissue states. Low-flow push–pull perfusion sampling (LFPS) has been used to collect submicroliter volumes of extracellular fluid which are well suited to capillary electrophoresis for compositional quantitative analysis. In this study, LFPS is used to deliver pharmacological agents to the in vivo retinal tissues at the probe sampling tip during sampling to measure protein function. Two native nitric oxide synthase enzymes were pharmacologically inhibited and the enzyme product NO metabolite, nitrate, was determined with capillary electrophoresis from the perfusates. LFPS delivered inhibitors including the non-selective N(G)-nitro-Larginine methyl ester (L-NAME), the nNOS selective 7-nitroindazole (7-NI), and eNOS N5-(1-imioethyl)-L-ornithine, dihydrochloride (L-NIO) were perfused to the sampling region either directly over a rat retina optic nerve head or 1-mm peripheral to the ONH. At the PONH, 65, 55 and 60% of baseline nitrate levels, respectively, were observed with inhibitor infusion. These are statistically significant ($P < 0.05$) compared to saline drug infusion. However, infusion of the inhibitors to the ONH did lead to significant nitrate concentration decreases. This data suggests that the endogenous enzymes, nNOS and eNOS, are both spatially and functionally localized to the PONH at the in vivo rat retina.

1. Introduction

Nitric oxide (NO) has been shown to regulate several homeostatic processes. More specifically, at the retina, it has been demonstrated as a key player in vasodilation [1], neurotransmission [2], inflammatory responses [3], and vasculature autoregulation [4]. Retinal damage can occur naturally and in diseases such as diabetic retinopathy [5,6] or glaucoma [7] due to an increase in the production of NO. Because of the relatively short half-life (4–6 s), it has become customary to assay the two stable metabolites, nitrate (NO_3^-) and nitrite (NO_2^-), as an indicator of the presence of NO [8]. With the use of a rat animal model and an *in vivo* sampling technique known as low-flow push–pull perfusion sampling (LFPS), we have reported an uneven distribution of the primary NO metabolite, nitrate, along various regions of the rat vitreous cavity [9]. Samples were collected and assayed for nitrate from three areas: the optic nerve head (ONH), regions 1–2 mm peripheral to the optic nerve head (PONH), and the middle vitreous (MV) region. The highest levels of nitrate were found at the PONH and the lowest levels were at the ONH. Because nitrate is the primary metabolite of NO, the ability to characterize such different levels of nitrate is quite surprising. The reported diffusivity of NO through biological tissue (3300 $\mu m^2/s$) [10] would not be expected to allow formation of significant concentration differences over such small tissue distances. We postulated that this asymmetric presence of nitrate could be associated with the distribution and function of the enzyme family, nitric oxide synthases (NOS), in the rat posterior chamber. Nitric oxide synthases are endogenous enzymes responsible for oxidizing L-arginine to NO and L-citrulline as a co-product [11,12]. In the central nervous system (CNS), the NOS subtypes have been shown to localize in neurons found in the cerebral cortex, corpus striatum, cerebellum, and the retina [13,14]. In the peripheral nervous system, NOS activity has been found in cell bodies and nerve fibers of the myenteric plexus and nerve

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processes in the posterior pituitary and adrenal medulla [13,14] as well as the cerebral blood vessels [15] and the choroid of the eye [13,14]. Three major NOS isoforms have been identified: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). The nNOS and eNOS enzymes are natively present and are calcium- and calmodulin dependent [16,17] whereas iNOS is calcium-independent and only expressed as an inflammatory response [18,19] thus the term inducible.

Efforts have been made to characterize the relative distribution of the NOS subtypes in the rat retina. One study has found that the constitutive NOS isoforms are primarily found in the peripheral ocular regions and minimally localize in the retinal nerve fiber layer or at the ONH in normal rats [20]. However, this study and similar ones were performed *ex vivo* where the animals were first euthanized then the retina was excised and examined. The retina is homogenized then subjected to western blot assays [7,20] or immunohistochemistry [7,21]. While these methods have provided information regarding the relative distribution of the enzyme family, there are a few disadvantages with them. First, because homogenates are used, those studies lack a high degree of spatial information about the distribution of the enzymes. Second, such assays require a degree of expertise to excise the retina and involve multiple steps which can be tedious and time-consuming. Furthermore, since the studies are performed *ex vivo*, one cannot rule out that the euthanization and excision process does not alter the localization of the NOS family. Finally, *ex vivo* results do not provide information about the functional properties of the enzyme.

Fortunately, LFPS is capable of providing data that is distinct from *ex vivo* techniques. Low-flow push–pull perfusion sampling has been applied to sampling from the rat brain [22–24] and eye regions [9,25] and has been described in detail in previous publications. Briefly, LFPS consists of fused silica capillaries aligned in a concentric design to allow simultaneous infusion of a desired solution, often Krebs-Ringer buffer (KRB), and withdraw of a sample from the end of the sampling probe. Samples are collected every 10 min into a replaceable tygon tube junction yielding sample sizes of ~500 nL. LFPS permits the ability to monitor endogenous or induced chemical composition changes through time. The infusion line of the sampling probe can be easily connected to an infusion syringe containing a different solution. This allows administration of pharmacological agents to induce changes in the physiology of the chemical environment that is being sampled from. Because sample volumes are relatively small (submicroliter), it is ideal to couple this *in vivo* technique with capillary electrophoresis with UV (CE–UV) detection. A rapid CE–UV assay was developed to assay nitrate in human [26] and rat vitreous samples [9].

The objective of this study was to spatially and functionally define the *in vivo* distribution of the NOS subtypes at the normal rat retina. Our goals were to pharmacologically manipulate the presence of the native NOS enzymes using LFPS and determine if the nitrate signal at different regions of the retina would be altered due to the delivery of an NOS inhibitor. The use of this methodology provides data to describe NOS activity at *in vivo* retina.

2. Experimental

2.1. Chemicals and reagents

Calcium chloride, magnesium chloride, sodium phosphate, sodium monobasic phosphate, sodium citrate, and citric acid were purchased from Fisher Scientific (Itasca, IL). Potassium chloride, sodium chloride, dimethyl sulfoxide (DMSO), N(G)-nitro-L-arginine methyl ester (L-NAME), cetyl trimethyl ammonium chloride (CTAC), sodium nitrate (≥99.0% m/m), and sodium nitrite (≥99.0% m/m) were obtained from Sigma-Aldrich (St. Louis, MO). 7-Nitroindazole (7-NI) and N5-(1-imioethyl)-L-ornithine, dihydrochloride (L-NIO) were purchased from A.G. Scientific (San Diego, CA). Proparacaine hydrochloride, gentamicin sulfate, and tropicamide were obtained from Akorn (Buffalo Grove, IL). The composition of the KRB was 3 mM KCl, 145 mM NaCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.61 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, and 0.2 mM ascorbic acid (pH 7.5). All solutions, unless stated otherwise, were prepared in deionized water from a US Filter Purelab Plus water purification system (Lowell, MA) and filtered with a 0.22 μm Millex GP filters purchased from Millipore Corporation (Bedford, MA).

2.2. Animals

Male, Hooded-Long Evan rats weighing 300–600 g were purchased from Harlan. Animal protocols were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee and followed the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. Rats were allowed to adapt to their controlled environment (25 °C and 12-h:12-h light/dark cycle) with food and water available *ad libitum* for at least one week prior to experimentation.

2.3. Sampling procedure and administration of nitric oxide synthase inhibitors

Animals were prepared and secured in the stereotaxic apparatus according to the method previously described [9,25]. Briefly, the animals were deeply anesthetized using pentobarbital and a local anesthetic was applied to the eye. After dilation was achieved, the LFPS probe was inserted into the posterior chamber of the rat eye via a 29-gauge (180/340 μm i.d./o.d.) guide needle. The probe tip was positioned either at the ONH or in regions PONH. Half microliter samples were collected every 10 min in the tygon tube junction. During the initial 60 min of sample collection KRB was infused to the sampling area to establish baseline values for nitrate. After the baseline period, the infusion line was connected a syringe that contained one of the NOS inhibitors. The inhibitor syringe contained either 5 mM L-NAME (non-selective), 7-NI (nNOS selective), or L-NIO (eNOS selective). The L-NAME and L-NIO inhibitors were prepared in KRB and 7-NI was prepared in KRB with 25% DMSO for solubility. The inhibitor was infused for 30 min before the infusion line was returned to the syringe that carried KRB.

2.4. Nitrate electrophoretic assay and data acquisition

After sample collection, the amount of nitrate found in each sample was quantitatively determined using the CE assay described previously [9,26]. The optimized CE–UV separation buffer consisted of 150 mM NaCl and 2 mM CTAC with the pH adjusted to 3.5 by 5 mM sodium citrate and citric acid. Peak heights were measured in raw electropherograms and converted to concentration using a constructed calibration curve. The measured concentration versus sampling time was plotted to determine what effect the inhibitor had on the observed nitrate values. Initial nitrate levels were likely high due to insertion of the guide needle [9,23]. A stable nitrate signal was seen 30 min after the probe insertion; therefore, samples 4–6 were used to calculate the baseline value of nitrate for the animals. Statistical calculations were performed in Microsoft Excel®. To determine if infusion of the inhibitor caused a significant decrease in the nitrate levels, one-way, repeated measures ANOVA were performed on data collected during the baseline period and the data points collected during drug delivery.