Published in final edited form as:

Nitric Oxide. 2010 January 1; 22(1): 51. doi:10.1016/j.niox.2009.11.006.

Variations of brain endothelial nitric oxide synthase concentration in rat and mouse cortex

R. Kenneth Czambel^a, Alexander Kharlamov^a, and Stephen C. Jones^{a,b,c,*}

^a Department of Anesthesiology, Allegheny-Singer Research Institute, Pittsburgh, PA 15212, USA

^b Department of Neurology, Allegheny-Singer Research Institute, Pittsburgh, PA 15212, USA

^c Department of Radiology, University of Pittsburgh, Pittsburgh, PA 15260, USA

Abstract

No information exists on the differences of eNOS concentration in brain tissue, [eNOS]_{br}, between animals during normal and hypotensive blood pressure and both between and within animals during moderate hypotension. To address these questions, we modified a commercially available enzyme-linked immunosorbent assay (ELISA) kit for determining murine [eNOS]_{br} since no method exists to measure [eNOS]_{br}. Optimization of the kit ELISA procedure using brain cortex homogenates from 3 normotensive rats and 1 wild-type and 1 eNOS^{-/-} (ko) mouse included recovery evaluation for each sample and the use of an “eNOS-free” homogenate calibrator diluent obtained from a mutant eNOS-ko mouse. Initial spike-and-recovery values of 12.5–27% suggesting a substantial sample matrix effect were improved with lipid removal treatment to 37.3% and to 70% with 1:20 dilution of the sample. Calibration standards prepared using eNOS-free buffer increased recovery values to 78% in micro-punch samples. The optimized ELISA was used in micro-punch (<1 mg) brain cortex samples from 6 hypotensive rats. Whole brain [eNOS]_{br} varied considerably from 5–11 fmol/mg wet weight and was different between normo- and hypotensive animals ($p = 0.023$). The variability of [eNOS]_{br} due to moderate hypotension in micro-punch rat brain cortex samples was composed of both between (24%) and within (76%) animal components. The differences and variability of [eNOS]_{br} between normo- and hypotensive animals, and between and within hypotensive animals suggests the potential utility of its measurement for investigations of cerebrovascular physiology and that [eNOS]_{br} itself could be an important factor in cerebrovascular regulation.

Keywords

ELISA; Brain tissue; Matrix effects; eNOS; Micro-punch samples; Hypotension; Hypotensive blood pressure

Introduction

Endothelial nitric oxide synthase (eNOS) is one of three well characterized isoforms of the nitric oxide synthase enzyme family (EC 1.14.13.39) [1]. In mammals, these enzymes catalyze the production of the multifunctional second messenger nitric oxide (NO) and L-citrulline from

* Corresponding author. Address: Department of Anesthesiology, Allegheny-Singer Research Institute, 320 East North Avenue, Pittsburgh, PA 15212-4772, USA. Fax: +1 412 359 3986. sjones@wpahs.org (S.C. Jones).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

L-arginine, O₂, and NADPH-derived electrons [2]. eNOS is an important component of the multiple cerebro-vasoregulatory mechanisms in hypotension [3–5] and ischemia [3,6]. Measurements of NOS enzyme activity are usually done *in vitro* under optimal cofactor conditions, which can obscure *in vivo* behavior, and are rarely if ever isoform specific. Principles of eNOS regulation such as its interaction with caveolae [7] and its phosphorylation [8,9] are important, but the amount of protein can also modulate function, as phosphorylation is not always associated with eNOS activation [10]. eNOS phosphorylation at Ser 1177 in humans (Ser 1179 in rats and mice) was associated with increased NO production during portal hypertension [11]. However, phosphorylation state measurements, primarily focused on Ser 1177 [12], can ignore the complex regulatory mechanisms that involve many other phosphorylation sites, both stimulatory and inhibitory. Increased immunoreactivity of eNOS in heart endothelium but not total heart eNOS expression was noted in a diabetic rat model [13]. The concept that the amount of eNOS by itself is an important component of cerebrovascular regulation can only be part of the full story, as this enzyme is highly and specifically regulated, indeed controlled, by many factors [12]. However, when more enzyme is present, these regulatory factors can act more effectively.

Substantial variations in the cerebral blood flow response to hemorrhagic hypotension have been noted as variations in autoregulatory height using laser speckle perfusion imaging [14]. We suggest that these between- and within-variations might be due to variations in brain tissue concentration of eNOS, [eNOS]_{br}.

To facilitate research involving the direct study of the regulation and expression of the eNOS enzyme a precise and accurate quantitative method of analysis is vital. The enzyme-linked immunosorbent assay (ELISA) technique offers exquisite analytical sensitivity and is a convenient tool for the quantitative measurement of proteins. Accordingly, ELISA methods have already been established for the measurement of eNOS from cultured endothelial cells [15,16], and from samples of murine lung and heart tissue by Meurer et al. [16]. However, to our knowledge, no report of any method for the quantitative measurement of eNOS from brain tissue currently exists.

The use of ELISA assays for tissue homogenates is limited by matrix effects, defined in immunoassay procedures as those effects which interfere with the detection of the analyte [17]. Matrix effects for eNOS in brain tissue are presumably caused by brain lipids, lipoproteins, proteins, and the high level of protein intricacy [18] that results from the brain's genomic complexity [19], coupled with the low mass fraction of endothelial cells contained in cortical brain tissue. For these reasons, the only commercial eNOS ELISA kit (R&D Systems, Inc., Minneapolis, MN) available is designed and intended to measure eNOS from human endothelial cell lysates. For this ELISA kit, we suspected that matrix effects associated with homogenized rodent brain tissue would complicate or obscure accurate quantification due to low recovery even more so than in cell lysates [16].

We adapted the commercially available ELISA kit to investigate variations in eNOS concentration in brain tissue, [eNOS]_{br}, and report the first measurements of [eNOS]_{br} from rat and mouse brain tissue and in multiple micro-punch (<1 mg) brain tissue samples. We propose that significant variations in [eNOS]_{br} exist between normo- and hypotensive animals and between and within animals during moderate hypotension.

Materials and methods

Animal preparation

All animal procedures were approved by the Allegheny-Singer Research Institute Animal Care and Use Committee and were conducted in accordance with National Institutes of Health

guidelines for proper animal care. Nine adult male Sprague–Dawley rats (250–350 g; Taconic, Germantown, NY) and one adult male wild-type (wt) mouse (strain C57BL/6J) and one male mutant eNOS knockout (ko) mouse (strain B6/129P2-NOS3^{tm1unc/j}, ~28 g, Jackson Laboratories, Bar Harbor, ME) were used. The animals were maintained on standard laboratory rodent chow and water available *ad libitum*. Two eNOS-ko mice were used to supply the eNOS-free brain homogenate buffer.

Normotensive whole brain cortices: sampling and homogenization procedure

For the ELISA optimization and validation studies in whole brain cortices, three rats, a wt mouse and an eNOS-ko mouse were sacrificed by decapitation following isoflurane anesthesia and the brains were removed and dissected on ice. Blood pressure during this procedure was assumed to be normal or slightly hypertensive. In several studies, MABP increased 10–15% after an abrupt increase in isoflurane concentration to 1.3–5% [20–22]. The cortical tissue from both hemispheres was carefully separated from the subcortical areas of the brain to form a single tissue sample for each animal. The tissue was weighed without delay and homogenized by sonication in 50 volumes of ice-cold lysis buffer per milligram of wet tissue. The lysis buffer contained 50 mM Tris–HCl (pH 7.6), 100 mM NaCl, 10 mM EDTA, 0.5% (v/v) Igepal CA-630, with protease inhibitors (Complete Tablets, Roche Diagnostics Corporation, Indianapolis, IN) added just before use. The cortical tissue homogenate was incubated for 30 min on ice, briefly centrifuged (300 g for 5 min at 4 °C) to remove any insoluble debris (no eNOS was detected in the pellet), aliquoted into clean storage vials, and immediately stored at –80 °C for subsequent measurement of the eNOS protein.

Moderate hypotension micro-punch tissue collection procedure

For the studies of [eNOS]_{br} heterogeneity during moderate hypotension using micro-punch samples, six male Sprague–Dawley rats were used. Rats were anesthetized with isoflurane (2.5% induction and 1.0–0.5% maintenance), 30% oxygen and balance nitrous oxide, intubated and artificially ventilated (Harvard Apparatus, model 683, South Natick, MA). Body temperature was maintained at 37 °C by a rectal probe and servo-controlled heat lamp (YSI 74, Yellow Springs, OH). One venous and two arterial femoral catheters were inserted. Arterial blood gases (PaCO₂, PaO₂, pH) were determined with a pH/Blood Gas Analyzer (iSTAT, Abbott Point of Care Inc., Princeton, NJ). Arterial blood pressure (both mean and pulsatile) was continuously recorded on a polygraph (model 2600S, Gould, Inc., Cleveland, OH) and stored on the computer hard drive by a data-acquisition system, Windaq (Dataq Instruments, Akron, OH, USA). Pancuronium (Gensia Sicor Pharmaceuticals, Irvine, CA) was injected hourly (0.2 mg, IM).

For the collection of micro-punch brain tissue samples, rats were placed in a stereotaxic frame. Hemorrhagic hypotension to a target MABP of 70 mm Hg was induced by blood withdrawal from the femoral artery catheter into a heparinized 20 mL syringe over a period of ~10 min [14].

Rats were sacrificed by air emboli. Using a stereomicroscope, the skull was chipped away, the dura was removed to create an opening to the cortex, and the head of the rat was packed in dry ice. By direct application of 1 mL volumes of dry ice-chilled 2-methylbutane, the brain tissue was cooled to –14 °C as monitored by a thermal probe inserted into the brain.

Stereomicroscope-directed brain tissue micropunches were selected from paired, homotopic regions of each cortical hemisphere using a modified Becton–Dickinson 20 gauge spinal needle with stylet [23]. Each micro-punch was expelled into a tared 0.5 mL vial, immediately weighed, placed on ice, and ice-cold lysis buffer (140 µL) was added to each vial. Samples were immediately frozen and stored at –80 °C until subsequent measurement of eNOS protein.

Enzyme-linked immunosorbent assay (ELISA) procedure

Aliquots of whole brain cortices and micro-punch brain samples were assayed with the modified ELISA procedure described below with calibration standards prepared in either the kit calibrator buffer or eNOS-free “ko brain buffer.” Immediately prior to analysis, the thawed micro-punch samples were homogenized by sonication as described above, divided, and assayed in singlet: one portion was used for the ELISA assay and the other for a spike-and-recovery determination within the same assay. All assays were performed according to the manufacturer's protocol [24] with the following four modifications. (1) Each of the seven eNOS calibration standards was prepared as individual fractional dilutions in either the supplied calibrator diluent (a buffered protein base with preservatives) or the eNOS-free ko brain buffer diluent prepared from the mutant eNOS-ko mouse as described below, with concentrations ranging from 0 to 13.2 fmol/50 μ L. (2) The assay sample size was decreased from 100 to 50 μ L. (3) The first incubation time was increased to 18 h (overnight) and the temperature was decreased to 4 °C from room temperature to minimize the effects of sample loading time and to ensure that the capture antibody/antigen reaction proceeded to completion. (4) A spike-and-recovery determination was performed for each sample using a separate sample aliquot in a separate well. Absorbance at 450 nm was measured (EL321e Bio-Kinetics Reader, Bio-Tek Instruments, Inc., Winooski, VT) within 30 min of the addition of the “stop solution” (50 μ L 2 N sulfuric acid per well).

Whole brain cortex ELISA validation studies

Spike-and-recovery—To optimize recovery and evaluate the ELISA kit's analytical accuracy for use with brain cortex samples, spike-and-recovery experiments were performed. Using unadulterated (neat) aliquots from whole rodent brain cortices, samples were: (1) treated with a lipid-removing agent; (2) diluted by 20-fold; and (3) assayed with standards prepared with eNOS-free ko brain buffer as the diluent. A concentrated eNOS spiking solution (10.2 fmol/ μ L) was prepared using the lyophilized standard supplied (40 ng of a 19.7 kDa recombinant human eNOS fragment) by reconstitution with 200 μ L deionized water (Milli-Q Plus System, Millipore Corporation, Billerica, MA). The spiking solution was diluted 50-fold when added to the rat and mouse brain cortex homogenates to produce an enrichment of 10.2 fmol/50 μ L. The eNOS-supplemented homogenate samples were fractionally diluted, in five steps, down to a dilution of 1/20 using the kit's calibrator diluent and assayed in duplicate. All undiluted eNOS-supplemented samples were measured in quadruplicate. To further investigate matrix effects, a homogenized rat brain sample was diluted 20-fold using the calibrator diluent prior to the eNOS supplementation and ensuing fractional dilution described above. In addition, using only the lysis buffer, a spike control sample was prepared and assayed in the same manner as the spiked homogenate samples above to assess the compatibility of this buffer within the ELISA.

Dilutional linearity assessment—The analytical validity of these modifications was assessed with linearity of dilution experiments. Neat rat and wt mouse brain tissue homogenates were fractionally diluted, in five steps, down to a 2/5 dilution using the manufacturer's chosen sample diluent (the calibrator diluent). These samples were measured in the same assay as the spiked homogenate samples.

Sample treatment with a lipid-removing reagent—To assess possible assay interference by lipids present in the homogenate matrix, samples were treated with Cleanascite™ (Biotech Support Group, North Brunswick, NJ), a commercially available lipid removal and clarification reagent. This reagent is a saline suspension of a solid-phase non-ionic adsorbent (pH 8.0) that selectively removes lipids from biological samples. Immediately prior to use, the Cleanascite reagent was completely resuspended by gentle shaking. Cleanascite (200 μ L) was added to rat brain tissue homogenate (800 μ L) and mixed for 20 min

at room temperature by gentle shaking. Following centrifugation (1000g) for 20 min at 4 °C, the supernatant was carefully decanted into a clean collection vial and analyzed.

ko brain buffer preparation—Brain tissue from the eNOS-ko mice was prepared at a concentration equivalent to either the whole brain cortex lysate samples (20 µg/µL) or the micro-punch samples (5 µg/µL) for use as a diluent for the kit-supplied calibrator. To validate the use of eNOS-free ko brain buffer as a diluent for the calibration standards, these standards were prepared and assayed together with the kit's calibration standards.

Calculations and statistical analysis—The [eNOS]_{br} of each sample was calculated using a four parameter logistic transformation of the optical densities of the calibration standards corrected for background versus the concentration of the standards. The minimum detectable concentration was determined by adding three standard deviations to the mean optical density value obtained from 16 replicates of the zero calibration standard and interpolating the corresponding concentration from the standard curve. Linearity was assessed in relation to the [eNOS] determined from the standard curve. Linear regression analysis was used for the analysis of dilutional linearity and was evaluated using the coefficient of determination, R^2 . Recovery (%) was calculated as the amount observed minus the amount in the base all divided by the amount added times 100. eNOS concentrations were expressed as fmol/well or recovery-corrected [eNOS]_{br} in fmol/mg wet weight (fmol/mg-ww).

For the whole brain cortex study, ANOVA was used to identify [eNOS]_{br} differences between animals and paired or unpaired *t*-tests were used to compare results between different treatments. For the micro-punch study a mixed linear model was used to estimate variance components (within- and between-animal) using SAS Proc Mixed (version 9, SAS Institute, Inc., Cary, NC) and to compare the levels of [eNOS]_{br} between left and right samples assuming a compound-symmetry form in the repeated measurements of [eNOS]_{br}. Statistical tests were 2-sided. Cortical [eNOS]_{br} concentrations obtained during ELISA optimization in rats ($n = 3$) with normal MABPs were compared to the mean [eNOS]_{br} of the micro-punch cortical samples ($n = 6$) with MABPs of 70 mm Hg using an unpaired *t*-test. Results are expressed as mean $\pm$ standard deviation and $p < 0.05$ was assumed to infer statistical significant.

Results

Physiological status

The physiological parameters before the period of hemorrhagic hypotension were all within the normal range: pH 7.39 ± 0.10 ; PaCO₂ 40 ± 13 mm Hg; pO₂ 83 ± 25 mm Hg; and MABP 103 ± 4 mm Hg ($n = 6$). The mean MABP during hemorrhagic hypotension was 73 ± 2 mm Hg.

Analytical sensitivity

The minimum detectable concentration of eNOS was estimated to be 0.13 fmol/well when using the calibrator diluent supplied with the kit, and 0.37 fmol/well when using the ko brain buffer.

Dilutional linearity

Linear regression analysis revealed a highly linear relationship between the [eNOS]_{br} and the dilution factor for all of the rat and mouse brain tissue homogenates with R^2 values greater than 0.92, as presented in Table 1. Fig. 1 presents the dilutional linearity for the neat, spiked, Cleanascite-treated, and 1:20 diluted samples for rat #3, in addition to that of the spike control. Note that the maximum [eNOS]_{br} for the undiluted spike control is 10.2 ± 0.27 fmol/well at 1/ dilution = 1, which agrees with the spike amount of 10.2 fmol.

The standard curves generated by using the kit's calibrator diluent and from the ko brain buffer showed maximum absorbencies of 1.13 vs. 0.3 at 450 nm, respectively, a factor of $1.13/0.3 = 3.8$ (Fig. 2).

Recovery

The spike control sample showed a recovery value of $100.5 \pm 2.7\%$ (Table 1). The average recovery rate obtained after eNOS supplementation for the three brain homogenate specimens (rats #1, 2, and 3) was 18.7% (Table 1). The treatment to remove lipids from a rat brain tissue homogenate sample with Cleanascite increased the mean recovery rate to $37.3 \pm 0.3\%$ ($n = 4$). The recovery-corrected results obtained from the Cleanascite-treated (7.36 ± 0.55 fmol/mg-ww) and the neat sample (7.86 ± 0.14 fmol/mg-ww) did not differ ($p = 0.20$; $n = 4$, paired t -test). Although the mouse #1 [eNOS]_{br} values from the kit buffer and ko brain buffer were significantly different ($p = 0.02$), the values were similar, differing by 9%.

When the rat brain tissue homogenate samples were diluted 20-fold into the calibrator diluent prior to eNOS enrichment, the rate of recovery increased to $70.3 \pm 0.2\%$ (Table 1), equivalent to a factor of $70.3/18.7 = 4.3$. Compared to the kit buffer, the ko brain buffer increased the average spike-and-recovery value for the mouse #1 and #2 whole brain analysis from 12.6% to 53.2%, a factor of 4.2. Mean recovery in the micro-punch samples ($n = 115$) reached $78 \pm 16\%$, seemingly combining the increase from the ko brain buffer and that from the dilutional effect.

eNOS concentrations

Measurable amounts of eNOS were detected from all three neat rat brain tissue homogenates as well as in the wt mouse specimen (Table 1). As expected, no eNOS was detected from the eNOS-ko mouse brain tissue homogenate sample. Measuring these samples in quadruplicate produced acceptably low intra-assay imprecision, with a COV < 8%. The [eNOS]_{br} from the four animals (three rats and one mouse) was significantly different ($p < 0.0001$, ANOVA) indicating that inherent heterogeneity exists between animals.

In six hypotensive rats with micro-punch samples, mean [eNOS]_{br} for all samples ($n = 115$) was 6.76 ± 1.24 fmol/mg-ww. As expected, there were no differences ($p = 0.9$) between right and left brain cortex samples. The estimated between- and within-animal variances were 24% and 76%, respectively. The difference between animals as well as the excessive variability within the brain cortex for each animal, indicates that heterogeneity of [eNOS]_{br} exists both between, as above for the whole brain eNOS analysis, and within animals.

[eNOS]_{br} in the hypotensive blood pressure group was decreased to 71% of the normotensive group ($p = 0.023$) as shown in Fig. 3.

Discussion

The major accomplishments of this work are: (1) the establishment of a convenient analytical method for the measurement of [eNOS]_{br}; (2) the first report of brain cortex [eNOS]'s; (3) the demonstration of substantial variation between animals in whole brain cortex samples and both between and within animals in multiple micro-punch brain cortex samples; and (4) that [eNOS]_{br} differs between normo- and hypotensive animals. These *in vivo* results suggest that [eNOS]_{br} itself is a factor in its ability to produce nitric oxide and that concentration alone is important in this protein's function in cerebrovascular regulation. The concentration of eNOS, as a constitutively expressed protein, is commonly considered static. Our result, that there is considerable variation in concentration alone, could well complement the established concept that cerebrovascular regulation by eNOS is tightly controlled by many modulators [25]. Ours

and others' previous measurements of heterogeneity in the cerebral circulation's response to hypotension [14,26,27], presumably based on nitric oxide from eNOS [4,5], correspond to the within-animal (regional) (76%) and between-animal (24%) variations in $[\text{eNOS}]_{\text{br}}$ documented in this work. This heterogeneity of the response of CBF to hypotension was previously noted in separate animals [28].

The high analytical sensitivity of this immunoassay methodology enabled the measurement of eNOS from the small amounts of brain tissue acquired using the micro-punch sampling process. Our sensitivities of ~ 0.13 and ~ 0.4 fmol/well for kit and ko brain buffer, respectively, compare favorably to the detection limit reported by Meurer et al. [16] of 0.13 fmol/well for purified eNOS protein.

This commercially available two-site ELISA kit is designed and has been successfully used for measuring eNOS from human endothelial cell lysates [29–32]. It contains purified *E. coli*-expressed recombinant human eNOS as the calibration material and eNOS specific antibodies generated against a C-terminal fragment of human eNOS. The homology between the primary protein structure of mouse and rat eNOS to human eNOS is $\sim 90\%$. Therefore, it is not surprising that our data show these antibodies generated from human eNOS cross-reacting with both the mouse and rat eNOS proteins, however it should be noted that our calibration for $[\text{eNOS}]_{\text{br}}$ is based on the relative immunoreactivity of a human eNOS fragment to murine eNOS.

The low recovery values found with the crude brain tissue homogenates implies the presence of a large proportional matrix effect (i.e., systematic error) [33]. We attempted to reduce this effect by three different approaches, two of which were incorporated into the modified ELISA procedure. The first approach was Cleanascite treatment of brain tissue homogenate samples. The limited increase (from 27 to 37%) of the rate of recovery obtained after using this lipid-removing reagent suggests that: 1) lipids, which comprise approximately 12% of wet brain tissue mass [34], only contribute a portion of the negative interference observed from brain tissue homogenates; 2) it did not remove all the lipids, including the more complex lipoproteins; or 3) significant matrix effects remain due to other interfering substances, presumably proteins. Even though the recovery gains using this reagent were very modest, it did not alter the estimate of the eNOS concentration, indicating that it did not remove the eNOS protein from within the sample. The second approach was to dilute the sample 20-fold in the calibrator buffer. This high dilution creates a sample matrix that is much more akin to that of the calibration standards as well as diluting any interfering substances present to very low levels. The third approach was to prepare the calibration standards in the eNOS-free ko brain buffer, which by design, yields calibration standards that are in a matrix equivalent to that of the specimen's. The reduced optical density response, by a factor of 3.8 ($= 1.13/0.3$), as shown in Fig. 2, of the ko brain buffer corresponds to the ratio of the increased recovery obtained with this "matrix equivalent" calibrator system (mean 53.2% from Table 1) to the recovery using kit buffer standards (mean 18.7%) of $53.2/18.7 = 2.85$. In the micro-punch studies in rats #4-9, both of these approaches combined to improve the recovery to 78% and corroborated the importance of closely matching the matrix of the calibration standards to that of the test specimen. Even with this gain in recovery, we have also included in our modification a per sample estimate of recovery so that our final $[\text{eNOS}]_{\text{br}}$ could be considered the actual value.

Our values of $[\text{eNOS}]_{\text{br}}$ ranged between 5 and 11 fmol/mg-ww, considerably less than those estimated in murine lung and heart tissue by Meurer et al. [16] of 51 and 38 fmol/mg-w/w relative to human eNOS, suggesting that considerable variations between tissues exists.

Although matrix effects can arise from any immunoassay component or step, the sample matrix is frequently a source for this interference. Consequently, for many immunoassay methods,

using samples from an unsubstantiated type of specimen causes challenging interference effects that often compromise precision and accuracy [17] due to low and unmeasured recovery. Still, to suit unique research requirements, some investigators have used novel and unproven specimens in commercially available immunoassay systems, but fail to report any analytical validation studies [35–38]. Regardless, it must be realized that any change in the type of biological specimen requires analytical validation studies to guarantee reliable assay performance. Because the majority of immunoassay methods are designed and validated for use with serum, plasma, saliva, or urine samples, most of the information on the subject of sample matrix or interference effects has been published for these types of specimens [39]. There is a paucity of information concerning immunoassay matrix effects observed from tissue homogenates, especially homogenates of brain tissue, although Meurer et al. [16] in their assay of [eNOS] from lung and heart mention that “The decrease in both the linearity of detection and in the detection limit of eNOS [compared to the analysis of cell lysates] is likely due to interference from components of the cell lysate.” One of the positive attributes of our modified method is the inclusion of a separate measurement of recovery for each sample. The disadvantage of this inclusion of recovery, especially for the micro-punch implementation, is that more sample is needed, but the advantage of eliminating the loss due to even acceptable recoveries of ~80% far outweighs this disadvantage. By evaluating recovery in each sample, an accurate estimate of [eNOS]_{br} can be obtained.

This immunoassay procedure was developed to complement ongoing laser speckle flowmetry studies of cerebrovascular regulation [14]. The usefulness of this immunoassay to analyze micro-punch samples taken from the same location that relative cerebral blood flow (CBF) as determined by laser speckle flowmetry is greatly dependent upon its analytical sensitivity. The minimum detectable concentration was statistically determined to be 1.47 fmol/mg-ww for the modified ko brain buffer calibration curve-based assay. This translates based on our dilutions, mean micro-punch weight, and recovery to an ability to assay [eNOS]_{br} down to 1.9 fmol/mg-ww, thus suggesting the potential usefulness of this immunoassay for the comparison of relative CBF and [eNOS]_{br}.

Our result, that concentration alone is important, could well complement the established concept that the cerebrovascular regulation via eNOS is tightly controlled by many modulators. The heterogeneity in the cerebral circulation's response to hypotension could potentially be further investigated by combining this quantitative micro-punch method with CBF laser Doppler flowmetry.

In conclusion, we have shown differences in [eNOS]_{br} between normo- and hypotensive animals and established its variability both between and within brain cortex using, to the best of our knowledge, the first method for the quantitative measurement of [eNOS]_{br}. Our data indicate that when calibration standards and the brain tissue homogenate samples share a sufficiently similar matrix, acceptable and correctable recovery values yield an ELISA that is useful as a research tool. Diluting the samples 20-fold with the calibrator diluent is not preferred because the resulting eNOS values are too low for adequate analytical precision. However, micro-punch samples with their low mass provide an appropriate dilution of 1:4 compared to our validation studies in whole brain cortex aliquots. Therefore, we recommend: (1) preparing eNOS calibration standards in a ko brain buffer; (2) using small (<1 mg) brain samples or samples of equivalent dilution; and (3) performing spike and recovery analysis for each sample to ensure accuracy for the estimation of eNOS concentration. These recommendations provide a useful modification of this ELISA kit for the measurement of eNOS from mouse or rat brain tissue homogenates.

Acknowledgments

The statistical assistance from Kirk Easley and Joseph Timpona's help with brain freezing are gratefully acknowledged. The support of the American Heart Association – Bugher Foundation, the Pennsylvania Tobacco Settlement, and National Institutes of Health Grants NS030839 and NS066292 is gratefully acknowledged.

References

1. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J* 1994;298:249–258. Review. [PubMed: 7510950]
2. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J* 2001;357:593–615. [PubMed: 11463332]
3. Huang Z, Huang PL, Ma J, Meng W, Ayata C, Fishman MC, Moskowitz MA. Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. *J Cereb Blood Flow Metab* 1996;16:981–987. [PubMed: 8784243]
4. Jones SC, Easley KA, Radinsky CR, Chyatte D, Furlan AJ, Perez-Trepichio AD. Nitric oxide synthase inhibition depresses the height of the cerebral blood flow-pressure autoregulation curve during moderate hypotension. *J Cereb Blood Flow Metab* 2003;23:1085–1095. [PubMed: 12973025]
5. Toyoda K, Fujii K, Ibayashi S, Nagao T, Kitazono T, Fujishima M. Role of nitric oxide in regulation of brain stem circulation during hypotension. *J Cereb Blood Flow Metab* 1997;17:1089–1096. [PubMed: 9346434]
6. Endres M, Laufs U, Liao JK, Moskowitz MA. Targeting eNOS for stroke protection. *TINS* 2004;27:283–289. [PubMed: 15111011]
7. Bucci M, Gratton JP, Rudic RD, Acevedo L, Roviezzo F, Cirino G, Sessa WC. In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat Med* 2000;6:1362–1367. [PubMed: 11100121]
8. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999;399:597–601. published erratum appears in. [PubMed: 10376602] *Nature* 1999 Aug 19;400(6746):792.
9. Sasser JM, Sullivan JC, Elmarakby AA, Kemp BE, Pollock DM, Pollock JS. Reduced NOS3 phosphorylation mediates reduced NO/cGMP signaling in mesenteric arteries of deoxycorticosterone acetate-salt hypertensive rats. *Hypertension* 2004;43:1080–1085. [PubMed: 14993198]
10. Fulton D, Harris MB, Kemp BE, Venema RC, Marrero MB, Stepp DW. Insulin resistance does not diminish eNOS expression, phosphorylation, or binding to HSP-90. *Am J Physiol Heart Circ Physiol* 2004;287:H2384–H2393. [PubMed: 15271670]
11. Iwakiri Y, Tsai MH, McCabe TJ, Gratton JP, Fulton D, Groszmann RJ, Sessa WC. Phosphorylation of eNOS initiates excessive NO production in early phases of portal hypertension. *Am J Physiol Heart Circ Physiol* 2002;282:H2084–H2090. [PubMed: 12003815]
12. Dudzinski DM, Igarashi J, Greif D, Michel T. The regulation and pharmacology of endothelial nitric oxide synthase. *Annu Rev Pharmacol Toxicol* 2006;46:235–276. [PubMed: 16402905]
13. Felaco M, Grilli A, De Lutiis MA, Patrino A, Libertini N, Taccardi AA, Di Napoli P, Di Giulio C, Barbacane R, Conti P. Endothelial nitric oxide synthase (eNOS) expression and localization in healthy and diabetic rat hearts. *Ann Clin Lab Sci* 2001;31:179–186. [PubMed: 11337908]
14. Kharlamov A, Brown BR, Easley KA, Jones SC. Heterogeneous response of cerebral blood flow to hypotension demonstrated by laser speckle imaging flowmetry in rats. *Neurosci Lett* 2004;368:151–156. [PubMed: 15351439]
15. Aberle S, Young TA, Medberry P, Parkinson J, Rubanyi GM, Kauser K. Quantitative measurement for endothelial constitutive nitric oxide synthase in cultured human endothelial cells. *Nitric oxide* 1997;1:226–233. [PubMed: 9704584]
16. Meurer J, Blasko E, Orme A, Kauser K. Quantitative measurement of endothelial constitutive nitric oxide synthase. *Methods Enzymol* 2002;359:433–444. [PubMed: 12481593]
17. Wood WG. Matrix effects in immunoassays, *Scand. J Clin Lab Invest Suppl* 1991;205:105–112.

18. Becker M, Schindler J, Nothwang HG. Neuroproteomics – the tasks lying ahead. *Electrophoresis* 2006;27:2819–2829. [PubMed: 16739225]
19. Velculescu VE, Madden SL, Zhang L, Lash AE, Yu J, Rago C, Lal A, Wang CJ, Beaudry GA, Ciriello KM, Cook BP, Dufault MR, Ferguson AT, Gao Y, He TC, Hermeking H, Hiraldo SK, Hwang PM, Lopez MA, Luderer HF, Mathews B, Petroziello JM, Polyak K, Zawel L, Kinzler KW. Analysis of human transcriptomes. *Nat Genet* 1999;23:387–388. [PubMed: 10581018]
20. De Cosmo G, Cancelli I, Adduci A, Merlino G, Aceto P, Valente M. Changes in hemodynamics during isoflurane and propofol anesthesia: a comparison study. *Neurol Res* 2005;27:433–435. [PubMed: 15949243]
21. Ishikawa T, Nishino T, Hiraga K. Immediate responses of arterial blood pressure and heart rate to sudden inhalation of high concentrations of isoflurane in normotensive and hypertensive patients. *Anesth Analg* 1993;77:1022–1025. [PubMed: 8214702]
22. Yli-Hankala A, Randell T, Seppala T, Lindgren L. Increases in hemodynamic variables and catecholamine levels after rapid increase in isoflurane concentration. *Anesthesiology* 1993;78:266–271. [PubMed: 8439021]
23. Yushmanov VE, Kharlamov A, Boada FE, Jones SC. Monitoring of brain potassium with rubidium flame photometry and MRI. *Magn Reson Med* 2007;57:494–500. [PubMed: 17326173]
24. R&D Systems. Quantikine, Human eNOS Immunoassay. Catalog No DEN00. 2003
25. Li H, Wallerath T, Förstermann U. Physiological mechanisms regulating the expression of endothelial-type NO synthase. *Nitric Oxide* 2002;7:132–147. [PubMed: 12223183]
26. Heimann A, Kroppenstedt S, Ulrich P, Kempfski OS. Cerebral blood flow autoregulation during hypobaric hypotension assessed by laser Doppler scanning. *J Cereb Blood Flow Metab* 1994;14:1100–1105. [PubMed: 7929652]
27. Todd, MM.; Warner, DS.; Maktabi, MA. Neuroanesthesia: a critical review. In: Longnecker, DE.; Tinker, JH.; Morgan, GE., Jr, editors. *Principles and Practice of Anesthesiology*. Mosby; St Louis: 1998. p. 1607-1658.
28. Jones SC, Radinsky CR, Furlan AJ, Chyatte D, Qu Y, Easley KA, Perez-Trepichio AD. Variability in the magnitude of the cerebral blood flow response and the shape of the cerebral blood flow-pressure autoregulation curve during hypotension in normal rats. *Anesthesiology* 2002;97:488–496. corrected. [PubMed: 12151941]
29. Cokic VP, Beleslin-Cokic BB, Noguchi CT, Schechter AN. Hydroxyurea increases eNOS protein levels through inhibition of proteasome activity. *Nitric Oxide* 2007;16:371–378. [PubMed: 17306993]
30. Koistinen P, Siitonen T, Mantymaa P, Saily M, Kinnula V, Savolainen ER, Soini Y. Regulation of the acute myeloid leukemia cell line OCI/AML-2 by endothelial nitric oxide synthase under the control of a vascular endothelial growth factor signaling system. *Leukemia* 2001;15:1433–1441. [PubMed: 11516104]
31. Dye JF, Vause S, Johnston T, Clark P, Firth JA, D'Souza SW, Sibley CP, Glazier JD. Characterization of cationic amino acid transporters and expression of endothelial nitric oxide synthase in human placental microvascular endothelial cells. *FASEB J* 2004;18:125–127. [PubMed: 14597568]
32. Senthil D, Raveendran M, Shen YH, Utama B, Dudley D, Wang J, Wang XL. Genotype-dependent expression of endothelial nitric oxide synthase (eNOS) and its regulatory proteins in cultured endothelial cells. *DNA Cell Biol* 2005;24:218–224. [PubMed: 15812238]
33. Feldcamp, CS.; Smith, SW. Immunoassay: A Practical Guide. Academic Press; Orlando: 1987. Practical Guide to Immunoassay Method Evaluation; p. 75-77.
34. Woods AS, Jackson SN. Brain tissue lipidomics: direct probing using matrix-assisted laser desorption/ionization mass spectrometry. *AAPS J* 8 (2006) :E391–E395.
35. Bruno MA, Cuello AC. Activity-dependent release of precursor nerve growth factor, conversion to mature nerve growth factor, and its degradation by a protease cascade. *Proc Natl Acad Sci USA* 2006;103:6735–6740. [PubMed: 16618925]
36. Chang CP, Huang WT, Cheng BC, Hsu CC, Lin MT. The flavonoid baicalin protects against cerebrovascular dysfunction and brain inflammation in experimental heatstroke. *Neuropharmacology* 2007;52:1024–1033. [PubMed: 17204294]

37. Mahmood A, Lu D, Chopp M. Intravenous administration of marrow stromal cells (MSCs) increases the expression of growth factors in rat brain after traumatic brain injury. *J Neurotrauma* 2004;21:33–39. [PubMed: 14987463]
38. Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann Neurol* 2005;57:67–81. [PubMed: 15546155]
39. Selby C. Interference in immunoassay. *Ann Clin Biochem* 1999;36(Pt 6):704–721. [PubMed: 10586307]

Fig. 1.

Dilutional linearity: eNOS concentration plotted against the dilution for the “spike control”, the brain tissue lysate buffer spiked with eNOS (solid red triangles) and four brain cortex samples from rat #3: three spiked samples, one at 1:20 dilution (purple squares), the other Cleanascite-treated (green diamonds), the spiked (blue circles), and neat (magenta triangles) samples. Note the high dilutional linearity for all types of samples, with coefficients of determination, $R^2 > 0.92$ for all samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Fig. 2.

Kit vs. ko brain buffer standards: the effect of matrix on standard curve data is shown by plotting absorbance values for the eNOS calibration standards prepared in the kit buffer matrix (manufacturers' recommendation) on the left ordinate, and for these same standards prepared in an "eNOS-free" matrix from a mutant eNOS-ko mouse brain tissue homogenate, ko brain buffer, on the right ordinate against their known values of [eNOS] on the abscissa. Both calibration curves show high linearity with $R^2 > 0.99$.

Fig. 3. [eNOS]_{br} differences between normo- and hypotensive rats: the [eNOS]_{br} (mean ± SD) is different ($p = 0.023$) between rats with normal blood pressure (rats #1–3, $n = 3$) and those that are moderately hypotensive (rats # 4–9, $n = 6$).

Table 1
Detection and recovery of eNOS from whole and micro-punch brain tissue samples during normo- and hypotension.

Sample ID	Blood pressure	Calibration standard matrix	[eNOS] _{br} ^a (fmol/mg-ww)	Mean [eNOS] _{br} [*] (fmol/mg-ww)	Recovery (%)	Dilutional linearity, R ²	
						Neat samples	Spiked samples
Spike Control		Kit buffer	NA		100.5 ± 2.7	NA	0.9998
Rat #1	Normotensive	Kit buffer	9.49 ± 0.07	9.55 ± 1.71	16.3 ± 0.5	0.9919	0.9574
Rat #2	Normotensive	Kit buffer	11.3 ± 0.41		12.6 ± 1.4	0.9861	0.9641
Rat #3	Normotensive	Kit buffer	7.86 ± 0.14		27.0 ± 1.6	0.9211	0.9946
Rat #3, Cleanascite-treated		Kit buffer	7.36 ± 0.55 ^{**}		37.3 ± 0.3	NA	0.9987
Rat #3, 1:20 dilution		Kit buffer	NA		70.3 ± 0.2	NA	0.9990
Mouse #1, wt		Kit buffer	11.3 ± 0.32		12.5 ± 1.1	0.9945	0.9875
Mouse #2, ko		Kit buffer	0		12.7 ± 0.3	NA	0.9995
Mouse #1, wt		ko brain buffer	10.2 ± 0.32 ^{***}		49.5 ± 4.4	0.9945	0.9941
Mouse #2, ko		ko brain buffer	0		56.9 ± 2.6	NA	0.9781
Rat #4	Hypotensive	ko brain buffer	5.57 ± 1.36	6.77 ± 1.18	103.1 ± 6.4	NA	NA
Rat #5	Hypotensive	ko brain buffer	7.78 ± 2.04		88.2 ± 7.2	NA	NA
Rat #6	Hypotensive	ko brain buffer	5.56 ± 0.88		78.8 ± 10.0	NA	NA
Rat #7	Hypotensive	ko brain buffer	7.96 ± 1.74		66.7 ± 9.1	NA	NA
Rat #8	Hypotensive	ko brain buffer	5.98 ± 3.35		64.2 ± 9.8	NA	NA
Rat #9	Hypotensive	ko brain buffer	7.76 ± 2.32		69.8 ± 7.3	NA	NA

Mean of kit buffer recoveries (rats #1–3) = 18.7%.

Mean of kit buffer recoveries (mice #1,2) = 12.6%.

Mean of mouse ko buffer recoveries (mice #1,2) = 53.2%.

Mean of rat ko buffer recoveries (rats #4–9) = 78.1%.

^aeNOS (mean ± SD, corrected for recovery) was detected in all three normotensive whole-cortex rat and wt mouse samples and in all hypotensive rat samples. Whole brain values were measured in quadruplicate (n = 4) and hypotensive samples were taken as the average of 16–20 cortical micro-punch samples.

* p = 0.023 between normo- (rats #1–3) and hypotensive rats (#4–9).

** p = 0.20 compared to rat #3, paired t-test.

*** p = 0.02 compared to mouse #1, kit buffer, paired t-test.