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Original article

Use of an electrochemical nitric oxide sensor to detect neuronal nitric oxide production in conscious, unrestrained rats

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Abstract

Introduction: Amperometric sensors that directly measure nitric oxide (NO) are readily employed in pharmacologic research. While several of these sensors have been developed, none has been investigated for use in conscious, freely moving animals. An approach was developed and validated for real-time quantitation of neuronal NO production in rats without restricting locomotor activity or other potentially useful behavioral endpoints. **Methods:** Male Sprague–Dawley rats were equipped with a femoral vein or intraperitoneal cannula. A guide cannula and an amperometric NO sensor were placed in the left and right hippocampus, respectively. Following recovery, rats received a 6-h intravenous infusion of saline, L-arginine (an NO precursor; 250 or 500 mg/kg/h), or incremental intraperitoneal 7-nitroindazole (an NO synthase inhibitor; 200-mg/kg loading dose and 100 mg/kg every 2 h). The sensor recorded NO production continuously and microdialysis samples were collected incrementally throughout the experiment. Griess assay analysis of microdialysate samples was compared to sensor readings in vivo. In vitro degradation of an NO donor also was used to validate sensor performance. **Results:** Exogenous administration of L-arginine resulted in incremental increases in the neuronal NO signal. A reduction in NO production was observed during administration of 7-nitroindazole, a selective neuronal NO synthase inhibitor. A significant correlation was observed in vitro between the Griess assay analysis, an indirect analytical approach, and the NO sensor readings. The lack of a strong correlation between these measures in vivo is consistent with the indirect nature of the Griess assay. **Discussion:** The current approach allows real-time determination of neuronal NO production in unrestrained rats. This model will be invaluable in evaluating pharmacologic issues regarding brain tissue NO synthesis, assessing brain NO synthase as a molecular target, and establishing the effects of pharmacologic agents on neuronal NO production.

Keywords: Griess assay; Neuronal nitric oxide; Nitric oxide sensor; Rodents

1. Introduction

Nitric oxide (NO) is an endogenous molecule that has been implicated in numerous physiologic phenomena, and more recently, in disease pathogenesis and pharmacology (Moncada, Palmer, & Higgs, 1991). The involvement of NO in processes governing inflammation, neurotransmission, immune system regulation, progression of heart disease, Alzheimer's, and other prominent diseases makes it of interest to pharmacologists and biochemists seeking to treat disease processes related to NO regulation (Cockrell et al., 2000; Forte et al., 1997; Gulbenkians, Uddman, & Edvinsson, 2001; Kubes, Suzuki, & Granger, 1991; Law, Gauthier, & Quirion, 2001). Specific quantitation of NO in vivo is essential in understanding the dynamics of NO production, and thus elucidating the physiologic and pathogenic roles of NO.

Due to the short half-life of NO, indirect measurements commonly have been implemented to evaluate NO production in various model systems. These approaches include measuring a by-product of NO production (L-citrulline), quantitating oxidation products of NO (nitrates and nitrites), or determining complexation products of NO (hemoglobin or hydrogen peroxide complexes) (Braman & Hendrix, 1989; Bush & Pollack, 2001; Giraldez & Zweier, 1998; Wennmalm, Lanne, & Petersson, 1990). While providing reasonable data, these approaches are labor intensive, introduce high potential for error, and lack specificity.

One recently developed approach to specifically quantify real-time NO production is through the use of amperometric

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sensors. These electrochemical NO sensors have been developed by several groups. A comprehensive review amperometric NO sensors has been compiled by Palini, Curruli, Amine, and Palleschi (1998). While these sensors have been validated and used extensively in in vitro systems, few studies have been conducted in in vivo systems (Kalinowski, Matys, Chabielska, Buczko, & Malinski, 2002; Malinski & Taha, 1992; Shibuki, 1990; Villeneuve, Bedioui, Voituriez, Avaro, & Vilaine, 1998). Furthermore, all of these whole animal studies have been conducted in anesthetized animals (Brovkovych, Stolarczyk, Oman, Tomboulian, & Malinski, 1999; Mochizuki et al., 2002). Evidence suggests that anesthetics alter NO production, particularly the constitutive forms including neuronal NO synthase (Ichinose, Huang, & Zapol, 1995; Keita et al., 2000; Mueller & Hunt, 1998; Tobin, Martin, Breslow, & Traystman, 1994; Zuo, Tichotsky, & Johns, 1996). In order to investigate physiological NO production without the confounding factors of anesthetic effects, a need exists to develop an approach to quantify NO in vivo in awake, freely moving animals. Using a previously validated in vivo NO sensor, a method was developed to quantify neuronal NO in a conscious, unrestrained animal.

2. Methods

2.1. NO sensor and recording system

The amperometric sensor (amiNO-700F-EH, Harvard Apparatus, Holliston, MA) employed in these studies had an external diameter of 0.6 mm, and consisted of the sensing element surrounded by a gas-permeable membrane. The active and inactive connectors were 3 and 14 mm in length, respectively. The electrical signal generated by the oxidation of NO at the electrode surface was captured with an inNO-T recorder (Harvard Apparatus) and relayed to a personal computer. The inNO software (Harvard Apparatus) was used to record and store the output.

The NO sensors were calibrated using nitrite solutions which were converted chemically to NO with potassium iodide in an acidic solution. Sensors were polarized in aqueous solution for a minimum of 12 h prior to calibration, implantation into animal, or placement into NO-generating solutions.

2.2. Animal preparation

Adult male Sprague–Dawley rats (300–370 g) were purchased from Charles River Laboratories (Wilmington, MA) and were housed in a temperature-controlled facility (72 ± 2°F). Animals were allowed free access to food and water and were maintained on a 12-h light cycle (7 a.m. to 7 p.m.). Animals acclimated for a minimum of 1 week prior to experimentation. All procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, and were conducted in accordance with accepted standards for laboratory animal care.

Rats were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (10 mg/kg). A silicone rubber cannula (Helix Medical, Carpinteria, CA) was implanted in the right femoral vein (for intravenous administration of test compounds) or into the peritoneal cavity (for intraperitoneal administration), and exteriorized to the dorsal neck region. An intracerebral guide cannula (Bioanalytical Systems, West Lafayette, IN) was placed in the left hippocampal structure and an amperometric NO sensor was placed in the right hippocampal structure (bregma −5.6, lateral ±5, and dv −3 [guide cannula] or −7 [NO sensor]) using a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Dental cement was used to keep placements stationary. Instrumented rats were maintained in a microdialysis apparatus (CMA Microdialysis, North Chelmsford, MA) allowing free locomotion. The NO sensor was connected to a polarizing device and suspended from the liquid swivel arm (Fig. 1). This device maintained an electrical charge across the sensor during postsurgical recovery. Heparinized saline (20 U/ml) was used to maintain the patency of the venous cannula during the recovery period. Animals were allowed a minimum of 36 h to recover prior to experimentation.

2.3. Experimental protocol

On the day of the experiment, the NO sensor was removed from the polarizing device and connected to the

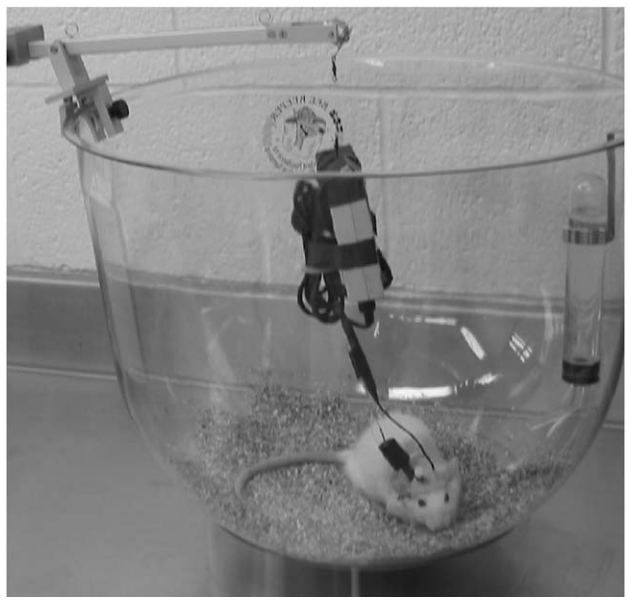

Fig. 1. Photograph of a fully instrumented rat. A swivel was attached to the horizontal arm extending from the bowl edge to the center, and allowed complete rotation of the animal. The polarizing device for the NO sensor is suspended from the swivel. A wire extended from the polarizing device to the rat and was attached to the rat collar. The horizontal arm was attached to a stationary piece with hinges and a spring to counterbalance the weight of the polarizing device. Counterbalancing allowed the animal to move up and down without placing stress on the implanted instrumentation.

inNO-T recording system which continued to maintain the polarized state of the NO sensor throughout the experiment. The insert was removed from the guide cannula and replaced with a microdialysis probe (BR-4, Bioanalytical Systems). The sizes of the microdialysis probe and the NO sensor were identical to ensure similar experimental conditions in both brain hemispheres (Fig. 2). The brain microdialysis probe was perfused at a rate of 5 μl/min with artificial cerebrospinal fluid (ACSF).

Four treatment groups ($n = 3–5$/group) were examined to assess the ability of the sensor to detect differences in neuronal NO production. L-Arginine, an NO precursor, was infused at a rate of 250 or 500 mg/kg/h into the femoral vein for 6 h, beginning at 9 a.m. The flow rate was maintained at 1.2 ml/h. Control rats received a saline infusion at an equivalent flow rate. The fourth experimental group received 7-nitroindazole, a selective neuronal NO synthase inhibitor, suspended in peanut oil and administered through an intraperitoneal cannula (200 mg/kg loading dose, followed by two doses of 100 mg/kg at 2-h intervals). Microdialysate samples (100 μl) were collected quantitatively every 20 min from the brain microdialysis probe, and hippocampal NO was quantified continuously throughout the experiment. Microdialysate samples were collected and NO concentrations were monitored for a total of 8 h.

Following experimentation, the animals were sacrificed, brain tissue was removed, and placement of the microdialysis probe and NO sensor was assessed visually to ensure proper hippocampal location. The dental cement was removed from the sensor using acetone and then soaked in a 0.25% trypsin solution at 37 °C for 30 min to remove proteins and tissue debris.

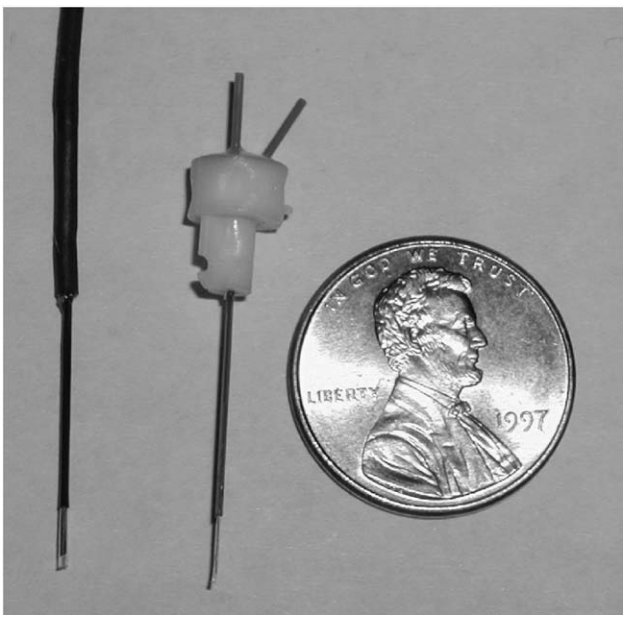

Fig. 2. Comparison of the size of the NO sensor (left) with the brain microdialysis probe (right). Penny shown to provide scale.

2.4. Indirect NO analysis

Indirect analysis of NO in microdialysate samples was used as a comparison with the performance of the NO sensor in vivo. NO was assessed indirectly by quantitating total nitrite and nitrate (NO_x^-) concentrations with a modification of the Griess assay (Bush & Pollack, 2001). Briefly, aliquots (50 μl) of microdialysate sample or nitrate/nitrite standard (1 μM in ACSF) were placed into wells of a 96-well plate. Nitrate was converted to nitrite enzymatically. NADPH (10 μl, final concentration 1 μM) was added to each sample, followed by a mixture containing glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and nitrate reductase (10 μl, final concentrations 0.5 mM, 160 mU/ml, and 80 mU/ml, respectively). The plates were then mixed and incubated at 25 °C for 1 h to allow conversion of nitrate to nitrite. Sample temperature was reduced to ~4 °C by placing the 96-well plate on ice for 30 min. Sulfanilamide and HCl (17 μl, 1 mM and 0.6 mM final concentration, respectively) were added, samples were mixed gently, and were incubated on ice for 15 min. *N*-(1-naphthyl)-ethylenediamine (NEDA) (17 μl; 1 mM final concentration) was added, samples were mixed gently, and were incubated at 25 °C for 30 min. Spectrophotometric analysis of samples was then performed on a fluorescence microplate reader (Biotek Instruments, Winooski, VT). Absorbance of samples was determined at 540 nm. Standard curves were constructed to verify that absorbance of the azo dye formed was proportional to the NO_x^- concentration.

2.5. In vivo validation in anesthetized animals

The NO sensor-generated signal is sensitive to noise associated with animal movement. To assess the extent to which this movement-induced noise might obscure the sensor reading, an in vivo validation was conducted in anesthetized animals. Rats ($n = 3$) were equipped surgically with an NO sensor in the right hippocampus and a brain microdialysis probe in the left hippocampus as in the preceding experiment. Prior to initiating the experiment, these animals received a single intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). NO sensor readings were recorded and hippocampal microdialysis samples were collected until the rat regained consciousness (approximately 2 h).

2.6. In vitro validation

As a comparison to the in vivo situation, the performance of the sensor was validated in vitro using Angeli's Salt (AS), a releaser of NO (Cayman Chemical, Ann Arbor, MI). AS dissociates to release NO in a pH-dependent manner with a half-life of 2.3 min at 37 °C in 0.1 M phosphate buffer at pH 7.4 (Fukuto, Hobbs, & Ignarro, 1993; Maragos et al., 1991). The NO donor (5 mg) was reconstituted in 1 ml 0.01 M

NaOH (final concentration 41 mM) and stored on ice to prevent spontaneous conversion to NO. The calibrated NO sensor was placed in ACSF (37 °C) and allowed to establish a stable baseline reading (approximately 10 min). An aliquot of the concentrated AS solution in NaOH was added to the ACSF to achieve final concentrations of 1000, 500, 100, 50, and 10 μM (10 ml final volume), thus initiating the conversion to NO. NO production was monitored following addition of the AS. Peak NO concentrations were recorded and a 100-μl aliquot was removed from the solution for analysis by the Griess assay as described previously.

2.7. Influence of surgical instrumentation on nociceptive responses

A central theme of this laboratory is the elucidation of factors that modulate opioid pharmacodynamics. Recent attention has focused on NO as a mediator of morphine tolerance. Thus, potential changes in an animal's ability to detect and/or respond to nociceptive stimuli secondary to implantation of the NO probe would limit the utility of this model. To address this question, nociceptive response was assessed prior to and following surgery using a standard hot-plate test (55 °C; Columbus Instruments, Columbus, OH). Licking of the hind paw or jumping was defined as behavioral endpoints, and the time to occurrence of either of these behaviors was determined. Tests were performed in triplicate prior to and after (>24 h) surgery. The first exposure to the hot plate for each test represented a habituation trial; nociceptive response was taken as the average of the second and third trials. A 40-s cutoff time was implemented to prevent tissue damage due to prolonged exposure to the hot plate.

2.8. Data analysis

Data collected from the NO sensor were smoothed using time averaging over 10-s intervals, followed by eigendecomposition to remove electrical interferences (Elsner & Tsonis, 1996). Autosignal Software (Clecom Software, Edgbaston, Birmingham, UK) was used to perform the smoothing and denoising algorithm. Orthogonal linear regression was used to establish a correlation between the Griess assay results and the NO sensor readings. Analysis of variance or Student's *t* test was used, as appropriate, to evaluate the significance of differences among or between experimental groups.

3. Results

The implanted sensor for analyzing neuronal NO production was well tolerated by the animals. Despite the extensive instrumentation required for the simultaneous analysis by the NO sensor and by microdialysis, rats recovered rapidly from surgery and displayed normal ambulation, feeding, and grooming behaviors by the time of experimentation. In addition, the hot-plate latency prior to and after surgery was not significantly different (7.6 ± 1.1 vs. 6.7 ± 1.0 s, respectively; $P>.05$).

Baseline noise in the NO signal was apparent and was associated with movement by the animal. A clean NO production signal was observed at all times when the animals were at rest or were stationary. Given the significant amount of data generated by the real-time determination of NO production, a signal-to-noise smoothing algorithm provided an effective means of filtering extraneous signals associated with locomotor activity. With this approach, analyzable data were obtained without restricting the movement of the animal, although some fluctuations in signal output (shorter term than the anticipated circadian variation in NO content) remained (Fig. 3).

Circadian variation in NO production was evident in both the NO sensor output and the Griess assay, as suggested by the time-dependent changes observed in the saline-treated animals. Exogenous administration of L-arginine, an NO precursor, at two different dose rates resulted in the anticipated incremental increase of neuronal NO (determined directly by the sensor and indirectly by the Griess assay of

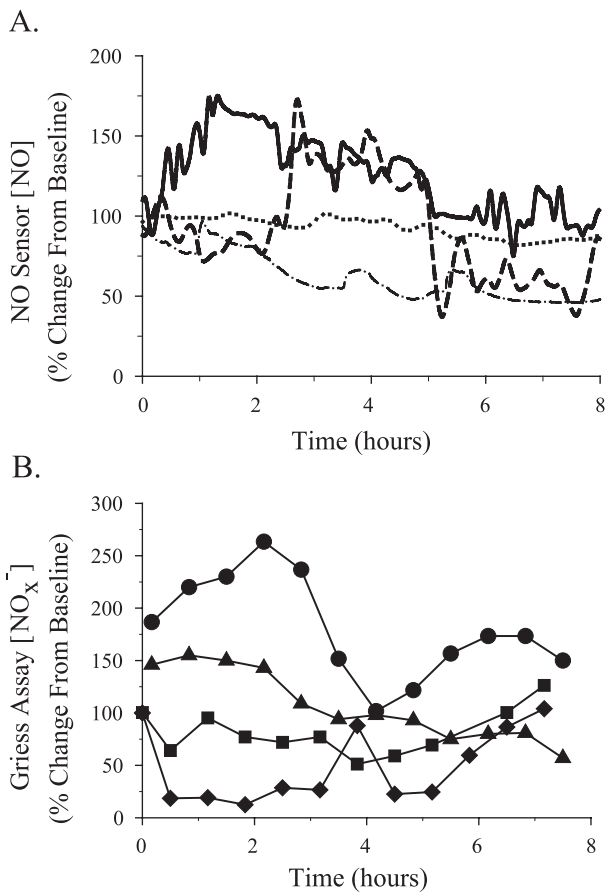

Fig. 3. Representative profiles of hippocampal NO concentrations as determined by: (A) the NO sensor, 7-nitroindazole (·--·), Saline (• • •), 250 mg/kg/h L-arg (- - -), 500 mg/kg/h (—), or (B) Griess assay, 7-nitroindazole (◆), Saline (■), 250 mg/kg/h L-arg (▲), 500 mg/kg/h (●).

NO_x^- in microdialysate). Furthermore, administration of a selective inhibitor of the neuronal isoform of NO synthase, 7-nitroindazole, reduced NO production when compared to the saline-treated group by both direct and indirect measures (Fig. 3). The apparent time-dependent changes in NO production remained during the treatment with either L-arginine or 7-nitroindazole. Although both analytical techniques indicated altered NO production in the presence of L-arginine and 7-nitroindazole, differences between the two methods were noted in terms of both the magnitude of change from baseline and the time course of changes in apparent NO content. In vitro, the two analytical methods were highly correlated, indicating that the degradation of NO to NO_x^- (in the absence of other sources of NO_x^-) allowed a reliable estimation of overall NO production (Fig. 4B). However, the relationship between the two assays was relatively poor in vivo both in the conscious (Fig. 4A) and anesthetized (Fig. 5A) rats, consistent with the fact that degradation of NO is not the only source of NO_x^- in the intact organism.

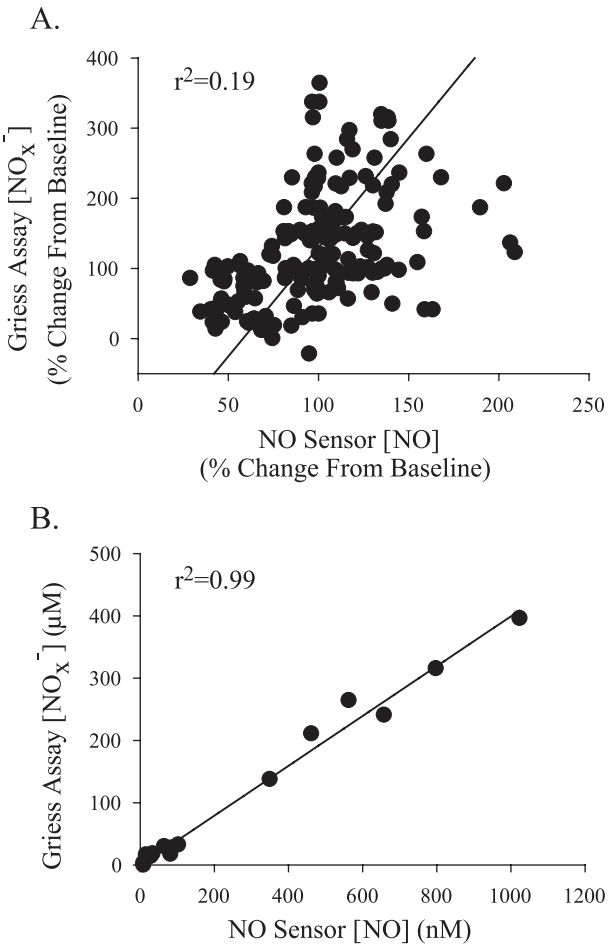

Fig. 4. Orthogonal linear regression of (A) in vivo and (B) in vitro (Angeli's Salt) NO production by the Griess assay versus the NO sensor. An improved correlation was observed when evaluated in vitro without the confounding factor of nitrate and nitrite diffusion across the blood–brain barrier.

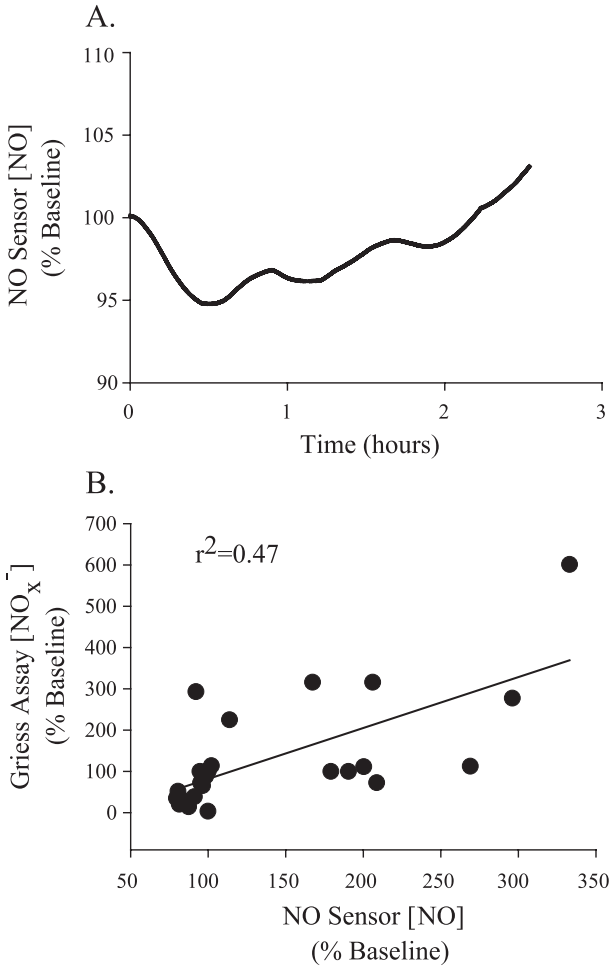

Fig. 5. (A) Representative profile of hippocampal NO concentrations (NO sensor) following intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). (B) Orthogonal linear regression of NO production by the Griess assay versus the NO sensor in an anesthetized animal (in the absence of movement-induced electrical noise).

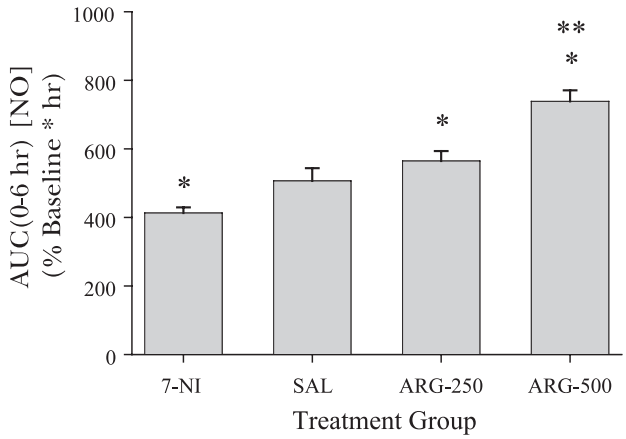

Fig. 6. AUC versus treatment regimen, 7-nitroindazole (7-NI), saline (SAL), 250 mg/kg/h L-arginine (ARG-250), 500 mg/kg/h L-arginine (ARG-500). AUC data are presented as mean ± S.E.M. ($n = 3–5$/group). * $P < .05$ when compared to saline-treated group, ** $P < .05$ when compared to ARG-250 treatment group.

Due to the large amount of data generated by this technique, as well as the time-dependent nature of NO production, it is reasonable to examine the overall brain NO content in response to treatment regimen, as opposed to the content at a specific, single point in time. To accomplish this, the area bounded by the NO sensor signal versus time profile (AUC) in treated rats provides a time-averaged measure that can be compared to control. Such AUC estimates are obtained readily with a linear trapezoidal estimation routine. This analysis of the data clearly illustrates the treatment effects on NO production consistent with the NO altering treatment regimen employed (Fig. 6).

4. Discussion

The current method for quantifying real-time neuronal NO production in vivo was successful, as suggested by several experimental observations. NO production clearly was elevated in response to administration of L-arginine, a precursor of NO, and was decreased in the presence of 7-nitroindazole, an inhibitor of neuronal NO synthase. Both the amperometric sensor and the Griess assay, used as a positive control for treatment-related effects on NO, indicated a dose-dependent increase in NO content in the hippocampus during L-arginine infusion and a reduction in NO content with administration of an inhibitor. It is unclear whether the minor fluctuations observed in the NO sensor outputs (Fig. 3A) were due entirely to random noise in response to movement of the animal or represent in part fluctuations in the production of NO in the brain. Given the extremely short in vivo half-life of NO and the role of NO in the inflammatory process, the observed oscillations could be due to minute movements of the sensor in the brain tissue eliciting an inflammatory response (Cockrell et al., 2000; Knowles & Moncada, 1994). Despite these slight fluctuations, a pattern of NO production was observed in the NO sensor output consistent with the NO-altering treatment regimen employed.

The circadian time course of NO production suggested by the present data is consistent with previously published observations on circadian variation in the activity of NO synthase and the corresponding protein levels in hippocampal tissue of rats (Ayers, Kapas, & Krueger, 1996). Enzyme activity and protein content were highest between 3 and 9 a.m., with a nadir in the late afternoon (Ayers et al., 1996). The data presented herein are consistent with this proposed 24-h cycle of NO production, and further indicate that the NO sensor is capable of detecting physiologically relevant changes in NO production.

A weak, albeit statistically significant, correlation was observed between the Griess assay analysis of microdialysate samples (for determination of total NO_x^-) and the NO sensor output (Fig. 4A). The Griess assay has been utilized extensively, and has been regarded as providing an accurate reflection of the time course of NO production (Bories & Bories, 1995; Feelisch & Stamler, 1996; Green et al., 1982; Nakahara et al., 2002; Ochoa et al., 1991). The fact that both techniques indicated increased NO production in response to L-arginine, and decreased synthesis with 7-nitroindazole administration, serves as an internal validation of the NO sensor. However, the large degree of variability in the correspondence between the two techniques in vivo, in light of the strong relationship in vitro (Fig. 4B), requires some consideration.

The Griess assay quantifies NO production indirectly by measuring total NO_x^-. This indirect approach is inherently nonspecific due to production of NO, with subsequent degradation to NO_x^-, by nonneuronal NO synthase, as well as other (non-NO related) sources of NO_x^- (Viinikka, 1996). For example, NO production by endothelial NO synthase should increase in response to L-arginine infusion. The NO produced in endothelial cells would degrade rapidly to NO_x^-, which would appear predominantly in the blood but would also diffuse freely into the brain. Due to the short half-life of NO, it is not possible that endothelially derived NO could activate the hippocampal NO sensor (with the exception of endothelial cells immediately adjacent to the implantation site) (Wood & Garthwaite, 1994). However, NO_x^- derived from NO produced at any endothelial site would have the opportunity to diffuse into the brain before being eliminated from the systemic circulation. Clearly, this alternate source NO_x^- in brain tissue would confound the NO_x^- concentrations obtained by brain microdialysis, making it virtually impossible to distinguish between NO_x^- derived from NO generated in neuronal tissue and NO_x^- arising from endothelial NO production. Measuring NO directly with the amperometric sensor eliminates this particular problem, as it measures NO only at the site of production.

The poor in vivo correlation could also be due in part to noise in the sensor output. To determine the degree to which this random noise might contribute to the lack of correspondence with the Griess assay, a comparison of NO_x^- in hippocampal microdialysate and NO sensor readings was obtained from anesthetized animals. Although these animals exhibited virtually no excess noise in NO sensor output (Fig. 5A), the correlation between the Griess assay and the NO sensor was similar to that in the in vivo study conducted in conscious ambulatory animals (Fig. 5B). Thus, the nonspecific nature of the Griess assay appears to be the primary source of the lack of correspondence between the two techniques.

While the sensor was clearly responsive to changes in NO production associated with the treatment regimen employed (Fig. 6), some consideration should be given to the possibility that the poor in vivo correlation could be caused by extraneous readings from the sensor due to oxidation of other molecules. The selectivity of the sensor is conferred through several mechanisms: (1) the carbon fiber electrode employed in the sensor oxidizes NO

rapidly compared to other molecules, (2) the sensor is coated with a negatively charged polymer which prevents oxidation of negatively charged molecules by repelling them away from the electrode surface, (3) a high voltage is applied to the sensor which, when NO is oxidized, produces a high electrical current, thus conferring selectivity over molecules which generate a low current at the voltage employed (Malinski & Taha, 1992). Molecules of carbon monoxide and hydrogen sulfide would not interfere significantly with the sensor output due to the relatively slow oxidation of these molecules when compared to NO (Taha, personal communication). Furthermore, no confounding effects would be expected due to the presence of nitrates, nitrites, or other negatively charged molecules due to the charged polymer coating surrounding the sensing element. This coating serves to keep negatively charged molecules from reaching the electrode surface (Malinski & Taha, 1992). Other oxidizable molecules may interfere with the NO-generated signal including tyrosine, cysteine, serotonin, dopamine, and norepinephrine. Upon oxidation, these molecules generate a relatively low electrical current when compared to that of NO at the voltage applied to the sensing element. Therefore, at physiological concentrations, these currents would not contribute significantly to the NO-generated amperometric signal (Brovkovych et al., 1999). Given the high selectivity of the sensor, the likelihood of nonspecificities in the NO sensor reading is low and does not likely contribute significantly to the poor in vivo correlation observed in these investigations.

Based on the present observations, the amperometric sensor can be used to accurately quantify neuronal NO production in an unanesthetized rats. The ability to extrapolate this method to detect endothelial or inducible NO in the peripheral tissues in mobile animals remains to be investigated. However, these data suggest that NO measurement in other tissues should be possible using a similar method of probe implantation followed by complete surgical recovery. The mechanical stability of the sensors used in this experiment suggests sufficient durability to withstand prolonged implantation in animals.

The new sensor employed in these studies was amenable for use in conscious rats. While signal noise was generated with animal movement, these background frequencies can be excluded, using the smoothing approaches described, to determine a profile of NO production in the brain; this appears to have significant advantages relative to earlier techniques. The development of the method to measure NO directly in the brain of a freely moving animal provides an invaluable tool to probe the role of NO in behavioral pharmacology. This model can be used extensively to establish the relationship of NO to physiological as well as pathogenic effects of NO, to assess NO synthase as a molecular target to treat and manage disease states involving NO regulation, and to establish the effects of medications on NO production in vivo.

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