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# Measurement of nitric oxide in hippocampal slices: induction with nitroso compounds and the effect of depolarization

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**Abstract.** Nitric oxide not only acts as a messenger for different physiological processes, but also mediates neurotoxicity associated with a variety of neurological disorders including epilepsy. The molecular mechanisms behind these actions are unclear. In this study, we aimed to detect relative amounts of NO released from rat hippocampal slices by chemiluminescence measurements under NMDA stimulation and spontaneous depolarization conditions. Hippocampal slices were preferred because of their functional integrity useful in simulating *in vivo* conditions. The reliability of the system was verified by administering increasing concentrations of a NO donor sodium nitroprusside in different redox milieu and a NO scavenger, carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxy PTIO). The redox versatility of NO allows interconversion from neuroprotective to neurotoxic species by a change in the ambient redox milieu. We have quantitated NO formed under NMDA stimulation and spontaneous depolarization conditions, and showed that depolarization increased NO formation and was excitotoxic for the neural tissue.

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**Key words:** nitric oxide, chemiluminescence, hippocampal slices, superoxide radical, depolarization, epilepsy

## INTRODUCTION

Nitric oxide synthase (NOS, EC 1.14.13.39) is an enzyme that exists in three isoforms each encoded by distinct genes (Moshage 1997). All isoforms catalyze the conversion of L-arginine to citrulline and nitric oxide (NO). NO is not only involved in different physiological, immunological and neurological processes but also participates in many pathologies (Beckman and Koppenol 1996, Zhang et al. 1996).

NO has been reported to mediate neurotoxicity associated with a variety of neurological disorders ranging from focal stroke to epilepsy (Lei et al. 1992). The molecular mechanisms behind these conditions are unclear. They are thought to involve voltage-dependent $Na^+$-channels, glutamate receptors and NO formation since selective inhibition of each provides neuroprotection (Strijbos et al. 1996).

Excessive activation of N-methyl-D-aspartate (NMDA) receptors is thought to be responsible from neurodegeneration through the generation of reactive oxygen species (ROS) (Lafon-Cazal et al. 1993, Monaghan et al. 1989). Glutamate triggers the opening of cation-permeable channels associated with NMDA receptors. The entry of calcium into cells through these channels stimulates NOS activity by binding to calmodulin, which is a cofactor of NOS (Dawson et al. 1993).

The animal or cell culture studies on NO metabolism are carried out using NOS inhibitor/activators or through indirect measurements of NO end products. In view of the vast interest in the pharmacological and toxicological properties of NO, sensitive methods are urgently needed to facilitate this area of research. Developing a method for *in vivo* sampling and detection of NO is challenging because of its short half-life (3-5 s in physiological conditions) and sub-micromolar concentrations (Zhang et al. 1996).

In this study we have aimed to detect relative amounts of NO released from hippocampal slices by chemiluminescence (CL) measurements (Kikuchi et al. 1993a,b). Hippocampal slices were preferred because of their functional integrity necessary in experiments based on *in vivo* simulation. The reliability of the system was verified by administering increasing concentrations of sodium nitroprusside (SNP), a NO donor and carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxy PTIO), a NO scavenger. Finally, we have quantitated NO released from hippocampal slices under NMDA excitotoxicity and spontaneous depolarization conditions.

## METHODS

### Materials

HEPES, lucigenin (bis-N-methylacridiniumnitrate), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), NMDA, SNP and ascorbate were obtained from Sigma (St. Louis, MO, USA). Carboxy-PTIO was from RBI (Natick, MA, USA).

### Experimental protocol

The study was approved by the local Animal Care and Ethics Committee. Twenty-five Spraque-Dawley rats (21-25 days old) were used. Rats were decapitated, their brains were quickly removed and slices of 400 μm thickness with an average weight of 6.86 ± 1.62 mg were obtained using a Campden tissue slicer. Hippocampi were isolated and then aerated with carbogen (95% $O_2$ + 5% $CO_2$) in artificial cerebrospinal fluid (ACSF) for 45 minutes at 25°C for functional recovery (8-10 slices in 50 ml ACSF). ACSF had the following composition: 125 mM NaCl, 3.75 mM KCl, 1.2 mM $NaH_2PO_4$, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.4 (pH adjustment was made following equilibration of the solution with carbogen). NO formation was sustained either by adding SNP to the incubation medium or by providing additional reducing conditions. After equilibration for 45 min, some slices were left as controls and the other were further incubated for 45 min in depolarizing ACSF (dACSF). The composition of dACSF was as follows: 79 mM NaCl, 50 mM KCl, 1.2 mM $NaH_2PO_4$, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose.

For NO measurements commercial luminol was purified by recrystallization. Firstly, luminol was dissolved in 1 N HCl and then treated with Norit. The pH of the resulting solution was adjusted to 3 with acetic acid. This procedure yielded a crude pale yellow powder which was dissolved in 1 N NaOH and decolorized again with Norit. Its maintenance at 4°C for 5 days produced a colorless powder and its 2 x recrystallization from water resulted in pure luminol sodium salt.

### Biochemical studies

After incubation, slices were transferred to the counting vials for CL measurements which were carried out at room temperature using a luminometer (Mini Lumat LB

9506, EG&G Berthold, Germany). One transverse and one longitudinal hippocampal section were placed in each vial. Counts were obtained at 15 s intervals, corrected for wet tissue weight and the results were expressed as the area under curve (AUC) for a counting period of 10 min (AUC of relative light units (rlu)/mg tissue).

CL detection of NO was based upon the reaction between NO and luminol-$H_2O_2$ (Kikuchi et al. 1993a,b). Chemiluminescent probe for NO detection contained 18 μM luminol, 150 μM desferrioxamine, 2.5 mM $H_2O_2$, 2 mM $K_2CO_3$. Superoxide radicals were quantitated using lucigenin at a final concentration of 0.2 mM (Küçükkaya et al. 1996). Luminescence was recorded after addition of each probe to vials containing slices in 3 ml of PBS-HEPES buffer (1.76 ml of 0.5 M $KH_2PO_4$ and 6.08 ml of 0.5 M $K_2HPO_4$ were mixed, diluted to 100 ml with distilled water and then to one liter with 0.9% NaCl containing 0.02 M HEPES).

The release of lactate dehydrogenase (LDH) from slices was measured in ACSF or dACSF incubation medium. Ten hippocampal slices of 200 μm thickness were incubated under carbogen aeration for 45 min in vials containing 3 ml ACSF or dACSF. Subsequently, the supernatants were obtained and LDH activity of supernatants was measured using a commercial diagnostic kit (Diasys, Germany).

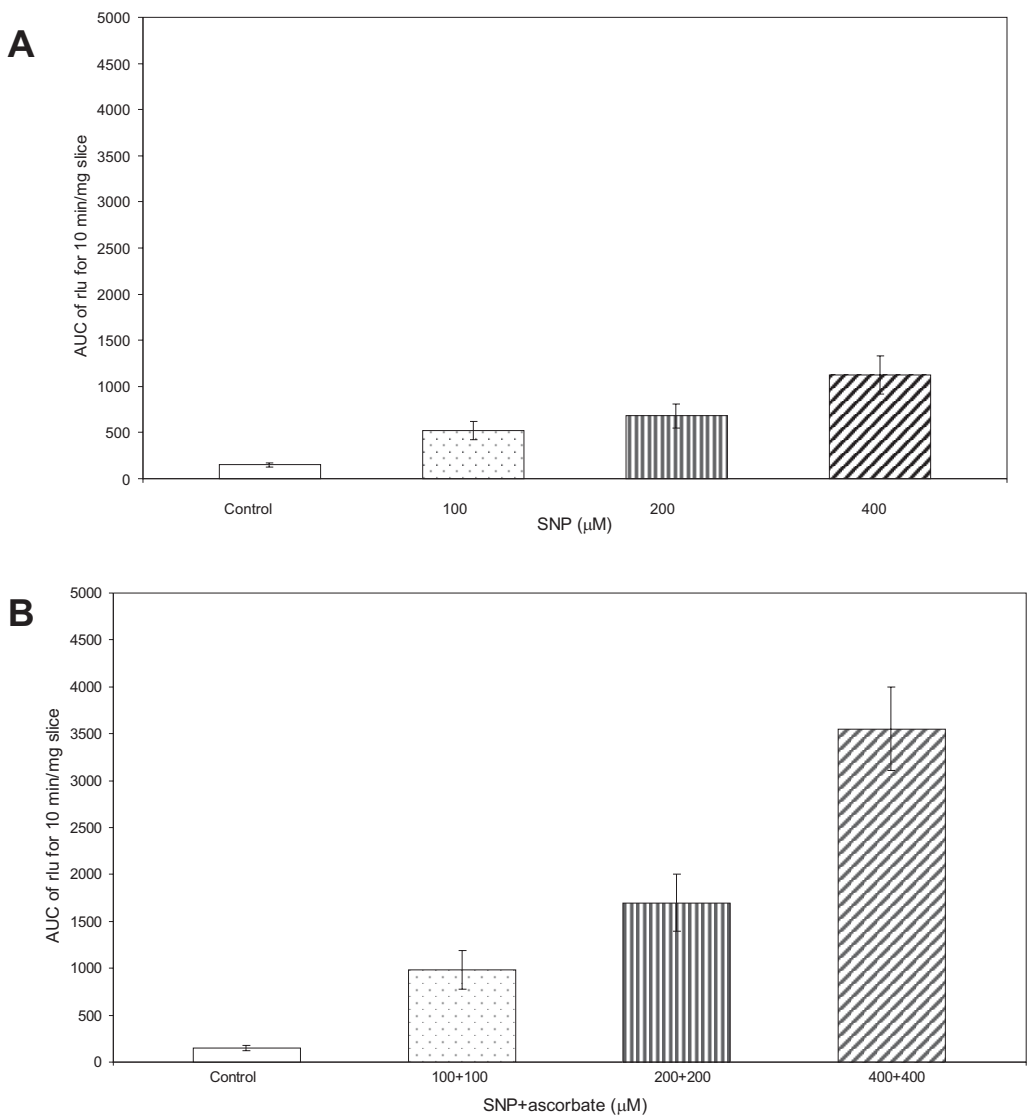


Fig. 1. Chemiluminescent measurement of NO in hippocampal slices ($n$ = 6) incubated with increasing concentrations of SNP alone (A) and both SNP and ascorbate (B).

### Statistical analysis

The significance of differences between the experimental groups were estimated by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison post-test. The differences were considered significant when the probability was $P<0.05$ (values were mean ± SD).

## RESULTS

Kikuchi et al. (1993a and 1993b) have demonstrated that NO reacts with $H_2O_2$ in the aqueous phase of the luminol-$H_2O_2$ system forming peroxynitrite which is a powerful oxidant yielding CL at both alkaline and neutral pH. Desferrioxamine is added to prevent hemoglobin interaction. NO derivatives such as nitrite and nitrate do not exhibit CL in this system, whereas thio-nitroso compounds exhibit $10^4$ times less emission than NO.

We have first examined the effect of exogenous SNP, a nitroso-compound which readily generates NO, in our measurement system. Increasing concentrations of SNP produced a dose-dependent response of NOS (Fig. 1A). SNP has a strong nitrosonium ion ($NO^+$) character which requires reductive activation to convert it into NO. Therefore, as shown in Fig. 1B, NO production was higher when equimolar ascorbate ions were added.

When carboxy-PTIO, a specific scavenger of NO molecule, was applied in its maximum inhibitory concentration (0.5 mM) to hippocampal slices treated previously with SNP, a significant reduction in CL signal (94%) was obtained suggesting that most of the signal was due to NO. Application of carboxy-PTIO to slices treated with SNP + Asc reduced the signal by 42%. These results imply that all $NO^+$ were converted to NO under reducing conditions. The deleterious effects of NO was evident by increased $O_2^-$. However, $O_2^-$ concentration was not significantly different from control slices when SNP was applied alone, since under these conditions $NO^+$ was neuroprotected by S-nitrosylation (Fig. 2).

We have finally investigated NO release from hippocampal slices under NMDA stimulation and depolarization conditions (Fig. 3). When 50 μM NMDA was added to the incubation medium of hippocampal slices, there was a slight increase in NO concentration which was not statistically significant ($P>0.05$). However, depolarization increased NO formation significantly ($P<0.05$ compared to both control and NMDA-stimulated conditions). LDH activity released from hippocampal slices into the incubation medium with ACSF was 167 IU/l. Under depolarization conditions LDH activity was increased to 294 IU/l.

## DISCUSSION

Interest in the measurement of NO production has increased enormously in view of the physiological functions of NO and the wide range of pathological conditions in which it has been implicated. Different

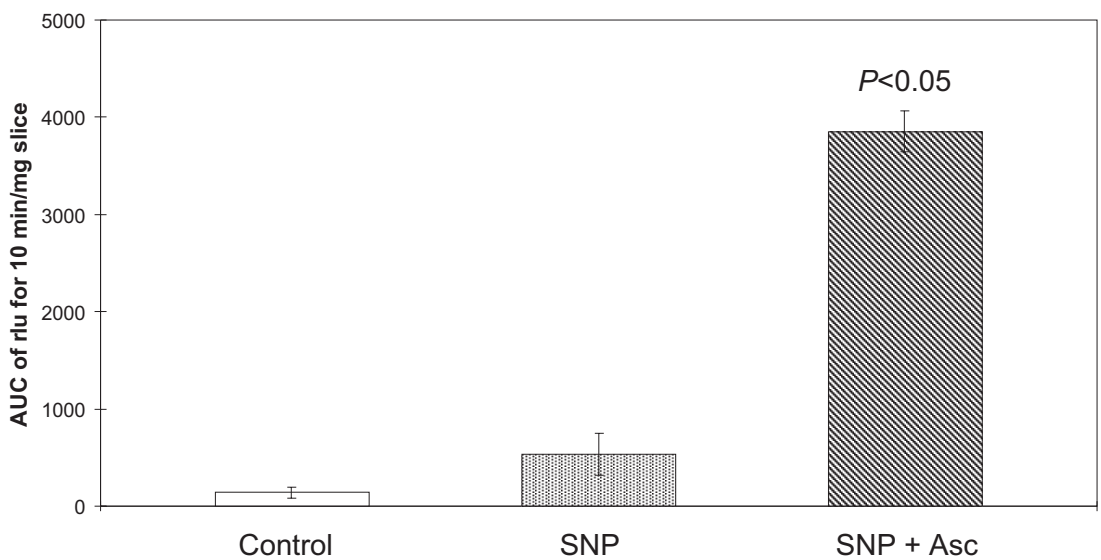


Fig. 2. Lucigenin chemiluminescence in hippocampal slices ($n = 6$) treated with SNP and ascorbate.

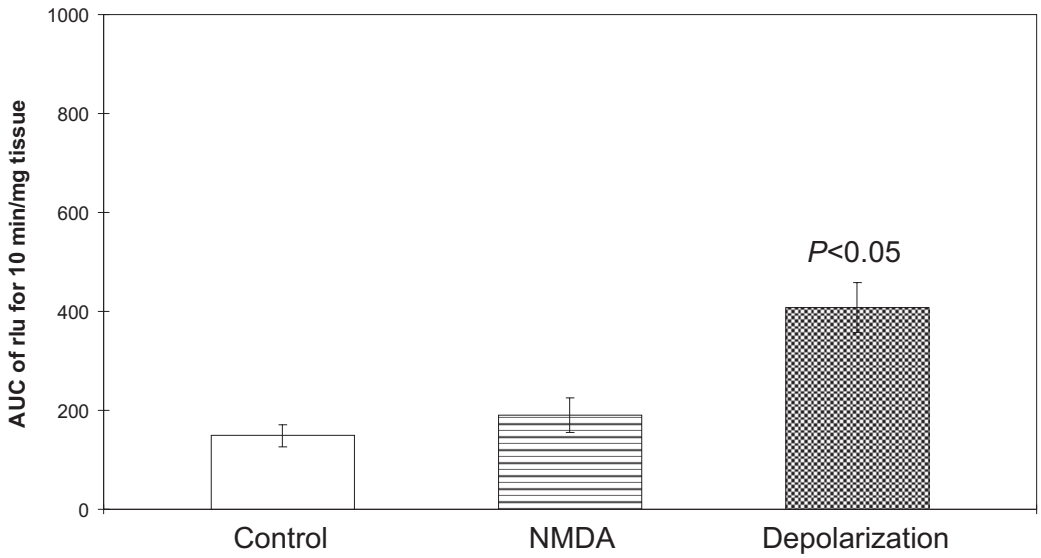


Fig. 3. Chemiluminescent measurement of NO in hippocampal slices ($n = 7$) after stimulation with NMDA and depolarization.

methods for determining NO and/or its metabolites (nitrite and nitrate) have been reported (Moshage 1997). "Real-time" NO determinations with microsensors can be applied to tissue explants, biopsies or isolated cells, the major disadvantage being the technical complexity (Vallance et al. 1995). Determination of the stable end-products is easy, but has limited sensitivity and specificity for the demonstration of the ongoing NO production (Zhang et al. 1996). EPR spectroscopy requires specialized equipment and expertise (Ohdan et al. 1995).

On the other hand CL measurement of NO is considered to be a useful method for sensitive and real-time assay of unstable radicals at low concentrations. Detection with luminol-$H_2O_2$ system was previously optimized for *ex vivo* organ perfusates (Kikuchi et al. 1993a,b). Our primary aim was to adapt the same system to hippocampal tissue samples. We have measured CL signals for 10 min and have expressed the data as the area under the curve (AUC). AUC was obtained by integrating the curve, which was constructed by counts taken within 10 min, by the trapezoidal rule (Allen and Loose 1976). The time interval for the measurements was selected such that slice viability was maximal.

We have tested the validity of the system by incubating slices with SNP, the NO donor. NO generated from donors or formed endogenously after NMDA receptor activation, can be either neurotoxic or neuroprotective depending on the changes in the ambient redox milieu (Dawson et al. 1991, Küçükkaya et al. 1996). The neurotoxic action of NO was correlated with the activation of NMDA ion channel activation and consequent $Ca^{++}$ influx (Lei et al. 1992). This situation leads to $Ca^{++}$-dependent stimulation of NOS and subsequent formation of other ROS (Allen and Loose 1976, Lafon-Cazal et al. 1993). The neuroprotective action is not mediated by NO itself, but occurs *via* S-nitrosylation of thiol groups by $NO^+$, which down-regulates the redox modulatory site of NMDA receptor and prevents excessive influx of $Ca^{++}$ (Brorson et al. 1997, Packer and Murphy 1994). Studies on different effects of NO are usually based upon indirect estimations of NO, use of NOS or $Ca^{++}$ channel blockers or determination of excitotoxicity by measurements of release of LDH in cell cultures (Dawson et al. 1993, Lipton et al. 1993).

In this study, we have used SNP which is a nitroso-compound with strong $NO^+$ character that readily generates NO, but can also be converted to NO by changing the redox milieu. When SNP was applied alone, both NO and $NO^+$ forms existed in the medium leading to S-nitrosylation and down-regulation of the NMDA receptor. Therefore, NO concentration was lower than that in which SNP was added together with ascorbate ions. The reducing environment produced by ascorbate converted all $NO^+$ to NO leading to neurotoxic stimulation of NMDA receptor and increased NO concentration by NOS activation. The ef-

fect of ascorbate in neurotoxic stimulation of NMDA receptor was also supported by high levels of lucigenin CL with SNP + Ascorbate compared to SNP alone.

In our reaction conditions, peroxynitrite release from slices cannot be distinguished from NO because the CL signal may be derived from peroxynitrite that is formed from either the conversion of NO to peroxynitrite by $H_2O_2$ and peroxynitrite that is already present in the slices. Application of carboxy-PTIO, a recently developed specific scavenger of NO, is useful in discriminating these two sources (Akaike et al. 1993, Nagase et al. 1997). When carboxy-PTIO was added to hippocampal slices incubated with SNP, nearly all of the CL signal dissappeared sugesting that the signal was due to NO formation. However, when it was added to SNP-Ascorbate incubated slices, only 42% of the signal disappeared. Rest of the signal was due to peroxynitrite formation from neurotoxic activation of $O_2^-$ and NO.

It has been accepted that upon NMDA excitation, cation permeable channels open, $Ca^{++}$ ions enter into the cell and stimulate NOS activity by binding to calmodulin (Dawson et al. 1993). Activation of NMDA receptor is also associated with increased generation of ROS, especially $O_2^-$ (Lafon-Cazal et al. 1993). The most probable mechanism for NO induced cell death is through peroxynitrite formed from reaction of NO with $O_2^-$ (Nagase et al. 1997, Pryor and Squadrito 1995). Addition of SOD/catalase prevented SNP-Ascorbate induced neuronal damage. This finding further supports the notion that neurotoxicity is caused by the reaction of NO with $O_2^-$ to form peroxynitrite (Lipton et al. 1993).

We have measured $O_2^-$ radical directly by lucigenin CL and have observed significantly increased levels in hippocampal slices incubated with SNP-Ascorbate. Gobbel et al. (1997) suggested that independent sources of NO and $O_2^-$ act synergistically to produce toxicity in cerebral endothelial cells. NO and $O_2^-$ contribute to the etiopathogenesis of several CNS disorders (i.e., epilepsy, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis). Different *in vitro* seizure models were developed using brain slices in which many synaptic connections are preserved. In hippocampal slices, seizures were induced by artificially elevating $K^+$ levels and changes have been shown to correlate well with *in vivo* seizures (Traynelis and Dingledine 1988). Elevation of $K^+$ produces membrane depolarization, which could promote NMDA receptor activation through relief of the $Mg^{2+}$ block and result in a larger and prolonged membrane depolarization (Mc Namara 1994).

We have measured the relative amount of NO released from hippocampal slices in the absence of NO donors. We first added NMDA at a low excitotoxic concentration, which resulted in a slight increase. However, NO release from hippocampal slices under depolarization conditions was significantly increased when compared to the slices incubated in ACSF. When interpreted together with increased LDH release from hippocampal slices after depolarization, these results imply that NO plays an important etiopathogenic role in epileptic seizures. It can be further suggested that depolarization increases NO formation which is excitotoxic for the neural tissue.

## CONCLUSION

In this study we have validated a previously described CL method for measuring NO in hippocampal tissue. Application of this method might enable elucidation of the pathophysiological role of NO in CNS disorders since neural tissue is prone to damage mediated by NO.

### ABBREVIATIONS

| | | |
|---|---|---|
| NO | - | nitric oxide |
| NOS | - | nitric oxide synthase |
| ROS | - | reactive oxygen species |
| SNP | - | sodium nitroprusside |
| carboxy PTIO | - | carboxy-2-phenyl-4,4,5,5-tetramethyl -imidazoline-1-oxyl-3-oxide |

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