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Fluorometric assay of nitrite and nitrate in brain tissue after traumatic brain injury and cerebral ischemia

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Abstract

Nitric oxide synthase (NOS) is distributed within the brain, and nitric oxide (NO) is felt to be involved in the pathophysiology of deterioration after head injury and cerebral ischemia. This study determined the levels of the stable end products of NOS (NO_x = nitrite + nitrate) after traumatic brain injury (TBI) and transient cerebral ischemia. A fluorometric assay using nitrate reductase and the NADPH regenerating system was used to quantitate NO_x in ultrafiltered (10-kDa cutoff) cortical and hippocampal extracts after reduction of nitrate. In TBI rats, both the plasma and tissue showed a sharp increase in NO_x levels 5 min after injury. Plasma NO_x returned to control levels by 2 h after injury. Ipsilateral-cortex NO_x levels returned to control levels ~6 h after injury and remained constant from 6–24 h. Contralateral-cortex returned near to control levels after 1 h. Hippocampus also followed a similar trend. In gerbils, there was a significant elevation in tissue NO_x levels immediately after 10 min transient cerebral ischemia, which gradually returned to control levels over 24 h reperfusion. This striking burst of NO synthesis immediately after injury is clearly evident whether the injury is head trauma or ischemia, or whether the measurements were performed on tissue or plasma. It is unknown whether endothelial NOS, neuronal NOS, or both caused the elevation of the NO end products seen after the CNS insults. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Traumatic brain injury (TBI); Cerebral ischemia; Nitric oxide synthase (NOS); NO end product; Nitrite/nitrate (NO_x); 2,3-diaminonaphthalene (DAN); Cortex; Hippocampus

1. Introduction

The increasing importance of nitric oxide synthase (NOS) has been underscored by the elucidation of its role in a growing number of normal and pathophysiologic processes [17,29]. Nitric oxide (NO), which is synthesized from L-arginine by NOS [7] not only plays a role in cerebral circulation, as an endothelium-derived relaxing factor [31,36,40], but also acts as a significant causative factor in the inflammatory response and neuronal injury after cerebral ischemia [2,17,21,22,24,25,36] and traumatic brain injury (TBI) [9,27,34]. Furthermore, glutamate excitotoxicity has been implicated in the pathogenesis of TBI and cerebral ischemia [6,8,11,23]. A presynaptic glutamate release causes postsynaptic calcium influx via *N*-methyl-D-aspartate receptors [8,38], which triggers several neurochemical reactions including NOS activation [7,13].

Direct measurement of NO in vivo is difficult due to its short half life [1]. NO rapidly decomposes into stable end products nitrite and nitrate. Quantification of NO_x (nitrite + nitrate) in biological fluids and tissue provides a useful method of indirectly estimating endogenously produced NO [1,12,24,25,34]. It is known that biological fluids such as plasma, cerebrospinal fluid, and urine have relatively large amounts of NO_x (μM–mM) which can be detected by Griess reaction [14]. However, cerebral tissue contains low levels of NO_x, for which the Griess reaction does not have sufficient sensitivity. Hence, a sensitive assay is needed to assess tissue NO_x levels.

Two methods based on sensitive fluorescence measurements using 4-hydroxycoumarin [30] or 2,3-diaminonaphthalene (DAN) [28] have been reported. The 4-hydroxycoumarin fluorescent product 3-amino-4-hydroxycoumarin is unstable under the assay conditions, unless the reaction is conducted at 0°C [30]. The hydroxycoumarin method applied to cerebral tissue measurements

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[21]: (1) determined only nitrite levels, (2) unfiltered crude tissue extracts were used, and (3) used 1 to 1.5 ml tissue extract for the assay. We selected the DAN method [28] due to the stability of the fluorescent product.

$$\underset{\text{(DAN, non-fluorescent)}}{\text{2,3-Diaminonaphthalene}} \xrightarrow[\text{HCl}]{\text{HNO}_2^-} \underset{\text{(fluorescent, stable at room temperature)}}{\text{2,3-diaminonaphthotriazole}}$$

Since these methods only measure nitrite, nitrate determination requires reduction to nitrite prior to assay.

The present method: (1) eliminated protein contamination by filtering the extracts through 10-kDa molecular weight cutoff filters to avoid interference from hemoglobin, (2) evaluated the conditions for the enzymatic reduction of nitrate, (3) determined NO_x in cerebral tissues by reducing the nitrate to nitrite using nitrate reductase and the NADPH regenerating system, (4) required only 10–40 μl of filtrate for the measurements. Since the tissue and plasma contain variable nitrite and nitrate levels, measuring NO_x levels gives more meaningful results.

In the present report, we have used nitrate reductase and the NADPH regenerating system, glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase (G-6-PDH) to reduce nitrate to nitrite and measured NO_x levels in plasma and cerebral tissue using DAN fluorescence. The novel aspect of this study, to the best of our knowledge, is that this is the first report measuring the time course of cerebral tissue NO end products (nitrite + nitrate = NO_x), an indirect measure of NOS activity and NO production, after TBI in rats and transient cerebral ischemia in gerbils.

2. Materials and methods

2.1. Materials

DAN was purchased from Aldrich chemicals (Milwaukee, WI). Nitrate reductase (EC 1.6.6.2), G-6-PDH (type IX) and other chemicals were purchased from Sigma Chemicals (St. Louis, MO). Centricon 10 filter units were purchased from Amicon (Beverly, MA).

2.2. Traumatic brain injury (TBI)

All surgical procedures were conducted according to the animal welfare guidelines set forth in the NIH Guide for the care and use of laboratory animals (US Department of Health and Human Services Pub 85-23, 1985) and were approved by the animal care committee of the University of Wisconsin-Madison. Male Sprague–Dawley rats (300–350 g) were anesthetized with 1.5% halothane in a mixture of 50:50 of N_2O:O_2. TBI was induced by using a controlled cortical impact device described earlier [3–5]. Each rat in the experimental groups was injured with a 6-mm diameter tip at a velocity of 3 m/s and 2 mm deformation while rats in the control group were subjected to the same surgical procedure, but received no impact. Mean arterial blood pressure and arterial blood gas levels were monitored and maintained within physiological limits. Core and cranial temperatures were monitored with rectal and temporalis muscle probes and maintained at 37–38°C for rectal and at 36–37°C for temporalis muscle with a heating pad and a lamp during experiments.

2.3. Transient cerebral ischemia

Male Mongolian gerbils (50–80 g) common carotid arteries were occluded for 10 min under halothane anesthesia and reperfused for various time periods as indicated according to Rao et al. [32,33]. Control animals underwent the same surgical procedure except the vessels were not occluded. Body temperature was maintained at 37–38°C during ischemia and reperfusion.

2.4. Sample preparation

2.4.1. Tissue

Animals were sacrificed, cortex and hippocampus from both hemispheres were dissected at 4°C and immediately frozen in liquid N_2. Tissue was homogenized in 20 mM Tris buffer, 10 mM EDTA (pH 7.4) and centrifuged at $3800 \times g$ for 20 min. The cytosol was assayed for Lowry protein.

2.4.2. Plasma

Blood was collected in heparinized tubes from TBI rats just prior to sacrificing the animals. Control blood samples were collected from uninjured rats, centrifuged for 5 min at $1200 \times g$ and plasma was collected.

2.4.3. Nitrite and nitrate assay

All the apparatus coming into contact with NO_x reagents was rinsed with double distilled water, since NO_x has been shown to be present even on new plastic and glassware [35]. Nitrite levels were determined by measuring the fluorescence of 2,3-diaminonaphthotriazole [28]. Since even 10 μM hemoglobin completely abolishes the 2,3-diaminonaphthotriazole signal [28], the cytosol and plasma were filtered through 10-kDa cutoff filters at $16\,000 \times g$ for 1 h. The filtered cytosol and plasma were used to assay NO_x levels. NO_x was determined following the reduction of nitrate to nitrite using nitrate reductase and the NADPH regenerating system (G-6-P/G-6-PDH). The optimal conditions for complete reduction were: nitrate reductase 30 mU; NADPH 3 μM; G-6-P 750 μM; G-6-PDH 48 mU in a final reaction volume of 100 μl and incubated for 90 min at room temperature in a 96-well microtiter plate. At the end of incubation, 30 μl DAN reagent (50 μg/ml of 0.62 N HCl), and after 10 min 30 μl of 1.4 N NaOH, were added to the incubation mixture. The fluorescence was measured at λ_{ex} 360 nm and λ_{em} 450 nm [28] using a Biolumin 960 UV/Fluorescence microtiter plate reader

(Molecular Devices, Sunnyvale, CA). Quantitation of nitrite or NO_x was based on external standards of sodium nitrite, or sodium nitrate reduced with reductase. For nitrite measurements the samples were not treated with nitrate reductase. Tissue NO_x levels were expressed as nmoles/mg of cytosolic protein. Plasma NO_x levels were expressed as μM.

2.4.4. Statistical analysis

All the measurements were expressed as means $\pm$ S.D. Data were analyzed using a one-factor ANOVA with the Bonferroni test to compare between the groups (GraphPad Software, San Diego, CA). A value of $p < 0.05$ was considered significant.

3. Results

We evaluated the effect of NADPH concentration and incubation time on NO_x assay. Fig. 1 shows the effect of increasing NADPH concentration on the nitrate reduction using nitrate reductase and the G-6-P/G-6-PDH regenerating system. Increasing NADPH concentration decreased the apparent nitrite concentration. For example, with 40 μM NADPH only $\sim$60% nitrite was observed. It appears that a high concentration of NADPH does not quench the fluorescence but may be affecting the nitrate reduction and DAN reaction (data not shown).

Fig. 2 shows the time course of $NaNO_3$ reduction with 3 μM NADPH using the G-6-P/G-6-PDH regenerating system. The reduction needed 90 min of incubation at room temperature to reach completion. Based on these results, samples were incubated with nitrate reductase for 90 min using 3 μM NADPH with the G-6-P/G-6-PDH regenerating system.

Plasma NO_x levels in control and TBI rats are shown in Fig. 3. Immediately after the injury the NO_x levels showed a dramatic increase within the first 5 min ($p < 0.05$).

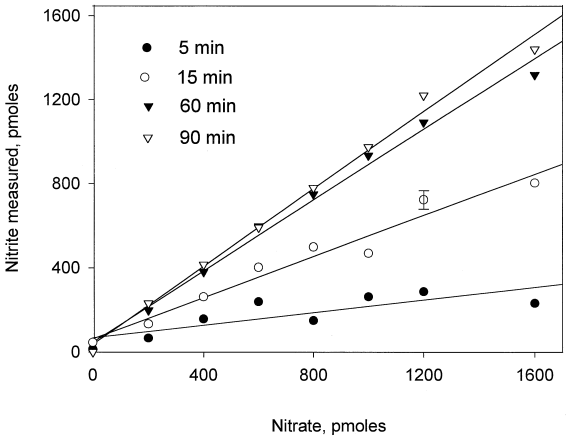

Fig. 2. Time course of nitrate reduction with 3 μM NADPH and G-6-P/G-6-PDH regenerating system. Samples were incubated at room temperature as described in Section 2. All the values represent the mean of three independent determinations. A representative error bar is shown.

These levels showed a rapid decline at 10 min, but continued to remain elevated upto 1 h and reached the control levels by 2 h. Sakamoto et al. [34] observed a similar pattern in extracellular NO_x levels using in vivo brain microdialysis in normothermic rats subjected to weight drop injury.

Tissue NO_x levels after TBI in the ipsilateral- and contralateral-cortices are presented in Fig. 4A. Injured group of animals were compared with the control group, and also ipsilateral-cortex was compared with contralateral-cortex. During the first 20 min after TBI, both ipsilateral- and contralateral-cortex NO_x levels were significantly elevated compared to the control group ($p < 0.05$). However, only the ipsilateral-cortex NO_x levels remained elevated significantly ($p < 0.05$) upto 2 h after TBI compared to the control group. Contralateral-cortex NO_x levels declined by 20 min after TBI then reached and remained at control levels from 1–24 h.

Between 1–2 h after TBI there was a significant ($p < 0.05$) difference in NO_x levels between ipsilateral- and

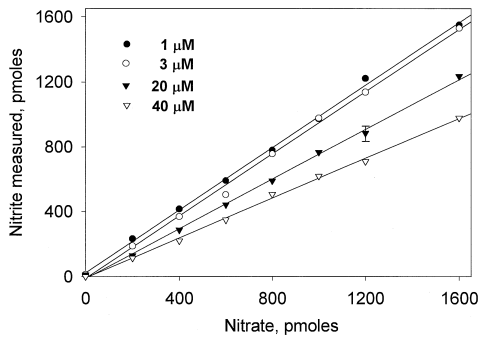

Fig. 1. Effect of NADPH concentration on nitrate reduction using the G-6-P/G-6-PDH regenerating system. Samples were incubated for 90 min at room temperature as described in Section 2. All the values represent the mean of three independent determinations. A representative error bar is shown.

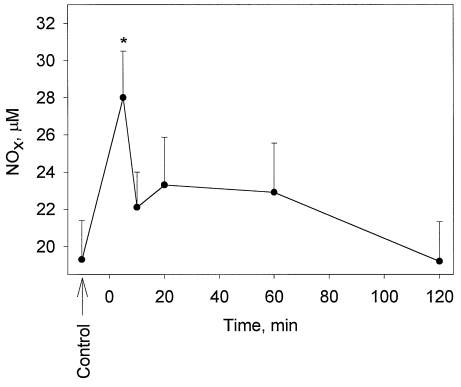

Fig. 3. Plasma NO_x in control and after TBI in rats. $*$ denotes $p < 0.05$. Comparisons were made between control and injury groups. Values are means $\pm$ S.D. ($n = 6$ per group).

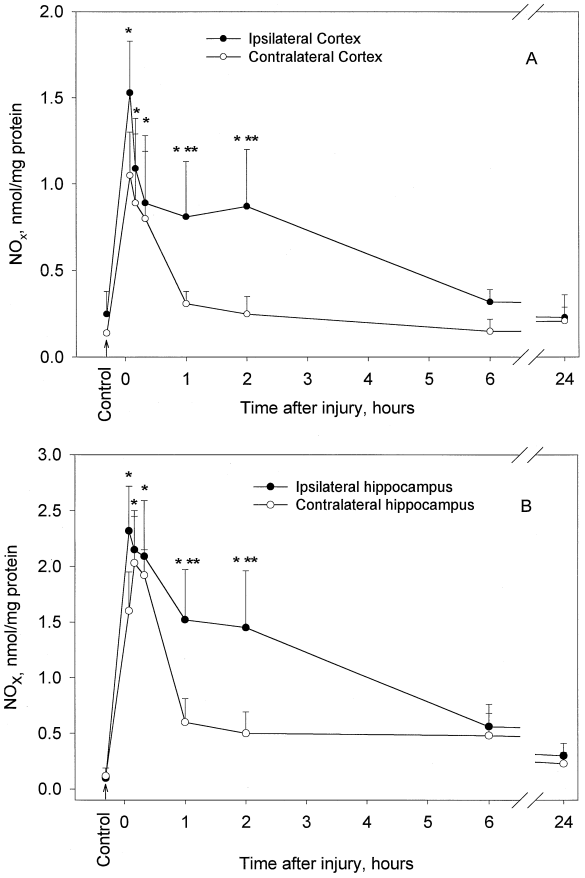

Fig. 4. (A) Cortical, and (B) hippocampal NO_x after TBI in rats. * denotes $p < 0.05$ for comparisons of control vs. injury groups. ** denotes $p < 0.05$ for comparisons of ipsilateral- vs. contralateral-cortices, or of ipsilateral- vs. contralateral-hippocampi. Values are means ± S.D. ($n = 6$ per group).

contralateral-cortices. By 6–24 h these differences were abolished.

Except for the absolute NO_x levels, the ipsilateral- and contralateral-hippocampi after TBI showed a pattern similar to ipsilateral- and contralateral-cortices (Fig. 4B). During the first 20 min after TBI, both ipsilateral- and contralateral-hippocampi NO_x levels were significantly elevated compared to the control group ($p < 0.05$). Similar to the cortex, the ipsilateral-hippocampus showed significantly higher NO_x levels at 1–2 h after TBI compared to contralateral-hippocampus ($p < 0.05$). These differences were abolished between 6–24 h.

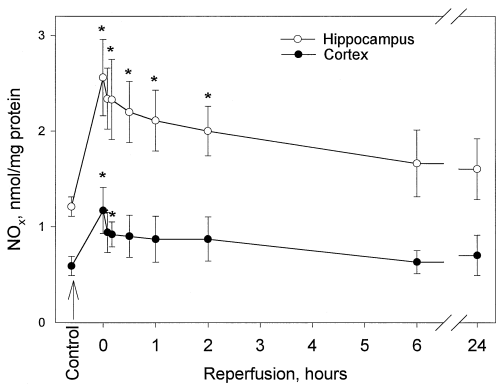

Fig. 5. Cortical and hippocampal NO_x after 10 min transient cerebral ischemia in gerbil with reperfusion over 24 h. * denotes $p < 0.05$ for comparisons of ischemic vs. control groups. Values were means ± S.D. ($n = 6$ per group).

Fig. 5 shows NO_x levels after 10 min of global transient cerebral ischemia and various reperfusion periods in gerbils. NO_x levels, both in the cortex and hippocampus immediately after 10 min ischemia sharply increased compared to the control group ($p < 0.05$). Over a 2-h reperfusion period the NO_x levels gradually decreased but still were elevated compared to the control group. These differences, however were only significant ($p < 0.05$) for the hippocampus from 30 min to 2 h. By 6–24 h they have almost returned to control levels.

4. Discussion

NO is a diffusible gas and is converted to nitrite and nitrate within seconds, thus it is difficult to directly measure changes in its concentration [1]. Measurements of the stable end products of NO, nitrite and nitrate (NO_x) provide a qualitative measure of NOS activity and NO production [12,26,29]. In the present studies, we measured NO_x levels in plasma and tissue after TBI and in tissue after transient cerebral ischemia. Nitrite levels were about 10–15% of the total NO_x (data not shown). Because of variability in nitrite and nitrate proportions in plasma and tissue, NO_x measurements provide a better indication of NO production, and hence only NO_x levels were reported. For example, in cerebral ischemia models, NO_x levels, but not nitrite levels, were elevated in plasma [24,25].

Since the tissue NO_x levels can not be measured by the Griess method [14], a sensitive method is needed. The fluorometric assay described by Misko et al. [28] used nitrate reductase with 40 μM NADPH and incubation for 5 min at room temperature for nitrate reduction. Recently it has been shown that high concentrations of NADPH affect the NO_x measurements. To minimize the effect of NADPH, a low concentration of $NADP^+$ (1 μM) was used with a regenerating system, G-6-P/G-6-PDH [39]. In this study, we first determined the optimal NADPH concentration and incubation time for complete nitrate reduction (Figs. 1 and 2) using the G-6-P/G-6-PDH regenerating system. Low concentrations of NADPH (1–3 μM) did not interfere with fluorescence measurements (Fig. 1). Using 3 μM NADPH concentration, we determined the optimal incubation time (Fig. 2).

In rat TBI, a sharp increase in plasma NO_x levels at 5 min after injury was observed (Fig. 3); a similar increase was also observed in cortex and hippocampus (Fig. 4A and B), suggesting an early burst of NO production after TBI. It is interesting to note that contralateral-cortex and hip-

pocampus between 5–10 min after TBI also showed an increase in NO_x ($p < 0.05$ compared to control) levels. In fluid percussion brain injury an early increase in lactate levels (an indication of the severity of injury) in both ipsilateral- and contralateral-cortices was observed. These levels remained elevated only in ipsilateral-cortex, whereas in contralateral-cortex these returned to control levels by 20 min after injury [10].

A possible explanation for this effect is that head injury results in a transient suppression of cerebral blood flow in the contralateral hemisphere [41]. Secondly, this intracranial pressure-induced circulatory arrest may cause metabolic changes which contribute to the increased NO_x levels in the contralateral brain [41]. Thus, the increase in NO_x levels in the contralateral-cortex and hippocampus in this study may be partly due to an induction of NOS by transient ischemia in the contralateral-cortex and hippocampus resulting from TBI. It is also possible that the tissue NO_x levels in the contralateral brain from 0 to 20 min may contain a small contribution from blood serum. The NO_x levels after 20 min may more accurately reflect tissue levels because: (1) serum levels declined rapidly after the injury, and (2) contralateral tissue levels reached near the control values from 1–24 h.

A similar early increase in NO_x levels was observed in global transient cerebral ischemia of gerbils (Fig. 5). These data are consistent with the observations reported by other groups. Thus, an early increase in NO_x levels was shown in plasma in focal cerebral ischemia [24,25], in in vivo brain microdialysate after contusion trauma [34] or transient cerebral ischemia of rats [37], and in tissue after permanent focal ischemia [2,21]. In gerbil global ischemia as well as in rat TBI, the hippocampal NO_x levels were higher than in cortex. These data are consistent with recent studies, which showed that hippocampus has more NOS activity as well as higher NO_x levels compared to cortex [35].

This striking burst of NO synthesis immediately after injury is clearly evident whether the injury was head trauma or ischemia, or whether the measurements were performed on tissue, plasma, or dialysate (Refs. [2,21,34,37], present study, Figs. 3–5).

In our studies, after the initial peak, NO_x levels declined rapidly in plasma and tissue in TBI but remained significantly elevated for 2 h in ipsilateral-cortex and hippocampus over the control group and over the contralateral-cortex and hippocampus. This may be partly due to increased glutamate release [22,34] leading to a post-synaptic calcium influx and activation of NOS [13] followed by systemic clearing of NO_x.

In transient cerebral ischemia, at least two factors may contribute to the elevated NO_x levels observed immediately after the 10 min ischemia (Fig. 5, 0 min reperfusion). In addition to NOS activation, some of this early accumulation of NO_x may result from decreased clearance from tissue during the ischemia due to local cerebral blood flow changes. Kader et al. also observed a bilateral increase in nitrite levels in hypothermic animals, which might have resulted from a decrease in cerebral blood flow [22].

The increase in NO_x levels suggests an increase in NO production. However the beneficial or deleterious effects of NO are unclear. It is known in the literature that NO produced by neuronal NOS (nNOS) is neurotoxic, whereas NO generated by endothelial NOS (eNOS) is neuroprotective [16,36]. Inducible NOS (iNOS) is localized mainly to neutrophils and vascular cells after ischemic injury [19] and requires up to several days for activation [18,20,42]. Thus, it is unlikely that the elevated NO_x in the present study were derived from iNOS. It is not clear from the present study whether nNOS or eNOS, or both, elevated the NO_x levels [34]. Further studies using eNOS [16] or nNOS [15] knockout mice or selective nNOS inhibitors [15] may clarify this issue.

In summary, a sharp increase in NO_x levels after TBI and transient cerebral ischemia suggests an increase in NOS activity and a resulting burst of NO production. The precise biochemical mechanisms underlying the activation of NOS as well as pathophysiological consequences of excess NO in trauma and ischemia remain to be clarified.

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