Electrochemical detection of nitroso-arginine as an intermediate between N-hydroxy-arginine and citrulline. An in vitro versus in vivo study using microcarbon electrodes in neuronal nitric oxide synthase and mice brain

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Abstract

The aim of the study was to describe in vivo and in vitro the transformation of N-hydroxy-arginine (NHA) into nitrite and citrulline. The products of NHA oxidation were studied by electrochemical methods. Cyclic voltammetry of NHA on microcarbon electrode showed an oxidation in two steps with one electron and one proton exchanged at each step. The first step gave a radical species NHA\(\cdot\) with a half-life shorter than 1 \(\mu\)s and the second step gave nitroso-arginine (NA) with a half-life of about 1 s (1.5 s). Coulometric oxidation of NHA gave citrulline and nitrite. Differential pulse voltammetry (DPV) in vivo and in vitro gave a peak in reduction at \(-1.66\) V vs Ag/AgCl for NA. After reductive adsorption of NA on the microelectrode surface in mice brain it gave the two peaks of NHA in oxidation plus another peak identified as nitrite. DPV in native and recombinant rat brain nitric oxide (NO)-synthase gave NA signal permitting \(K_m\) and \(V_{max}\) determination. All these results showed that NA was synthetized by NO-synthases before the final products, citrulline and nitrite.

Keywords: Nitric oxide; Nitrosoarginine; Voltammetry; Neuronal nitric oxide synthase; Brain; Mice

Nitric oxide (NO) was proposed a few years ago to be the active product of NO-synthases [5]. The detection of nitrite in biological tissues containing NO-synthases has been thought to reflect the presence of NO before oxidation to nitrite. The direct detection of NO has been done by electrochemical methods using microelectrodes [7,16]. In the cortex of rat brain the in vivo concentration of NO has been detected by amperometry with porphyrin microelectrodes at baseline and has been found to be 10 nM which is near the detection threshold of NO [8]. Amperometry in NO-synthases did not permit detection of NO without adding superoxide dismutase (SOD) [10,15]. Indeed, a NO-nitrosyl complex could be detected in NO-synthases without adding SOD by electron spin resonance (ESR) using a dithiocarbamate trap [21].

During the 80s, the true nature of endothelial derived relaxing factor (EDRF) was under debate in pharmacology. Comparative studies on the vasodilator effect of both NO and EDRF did not make clear whether these two compounds were the same [3]. Other compounds like hydroxylamine or nitroso-glutation are no longer considered as potential candidates for EDRF. Currently most authors suggest that the various effects of NO are related to its oxidation states [1,19]. Biological synthesis of NO was thought to be related to the oxidation of arginine which leads to an intermediate hydroxy-arginine (NHA) followed by the formation of nitrite and citrulline [20]. Furthermore it has been shown to be a substrate for NO-synthases [14]. The study of NHA oxidation provides some new insights into the second step of NO synthesis. Electrochemical methods which permit assessment of the number of electrons and kinetic parameters are also the best methods for the study of transient species as well as radicals during oxido-reduction reaction. Differential pulse voltammetry (DPV) with a microcarbon electrode indicated that NHA is oxidized to give nitroso-arginine (NA) in vitro [11]. Cyclic voltammetry on a platinum electrode in vitro showed oxidation in two steps [6].

The presence of NA has been hypothesized but never formally shown to be the direct precursor of NO [9]. A DPV peak attributed to NA has been detected in the rat brain, in aplysia neurons and is also contained in rat brain NO-synthase. In these preparations the concentration of NA...
increased with arginine and decreased with classical enzyme inhibitors [11–13].

In a first series of experiments cyclic voltammetry was performed with a potentiostat linked to carbon microelectrodes (Autolab, Roucaire, France). The sweeping rate was 40 mV s\(^{-1}\). Microelectrodes are commercially available (MFC1, Tacussel, France). Cyclic voltammetry of NHA (1 mg ml\(^{-1}\), in phosphate-buffered saline (PBS) pH = 7.4) on the carbon microelectrode is shown in Fig. 1. It presents two waves in oxidation. The electron and proton number exchanged during each reaction was equal to 1 unit. The two reactions were: NHA + 1e + 1H\(^+\) = NHA\(^-\) followed by NHA\(^-\) + 1e + 1H\(^+\) = NA (see Fig. 1). The half-wave potentials were +0.32 and +1.01 V vs Ag/AgCl for the first and second step, respectively. The calculated chemical reaction rates indicated a half-life of 1 \(\mu\)s or less for the radical species NHA\(^-\) and of 1.5 s for the formation of NA.

In a second series of experiments coulometry was performed in a microcell of carbon rod (0.141 cm\(^2\), Tacussel, France) in NHA solution (50 \(\mu\)l 10 g l\(^{-1}\), phosphate buffer saline 0.3 M pH = 7.4). The solution was in a closed box avoiding light during electrolysis at room temperature (20\(^\circ\)C). The additional potentiostat was a PRG5 (Tacussel, France) interfaced by AD/DA cards to a computer. Coulometry was performed during 1 h at different potentials: +0.4, +0.8, +1.2, +1.6 and +2 V vs Ag/AgCl. Oxidation and degradation products were analyzed by capillary electrophoresis which was performed by a P/ACE 5510 (Beckman, France) equipped with a photodiode array detector in a fused-silica capillary (75 \(\mu\)M diameter, 37 cm length). The electrolyte buffer was formic acid/formate (25 mM, pH = 2.5). The potential was set at 30 kV and the detector was set at 190 nm with 3D acquisition (190–600 nm). The oxidation of NHA gave nitrite, nitrate and citrulline. The results are presented in Table 1. The presence of nitrate was due to the oxidation of nitrite at the electrode surface. A compound X was found by capillary electrophoresis (Fig. 2) corresponding to the product of nitrosation of NHA by nitrite in acidic medium. It has previously been mentioned as the nitroso-adduct of NHA [17,18]. These results demonstrate that the electrochemical oxidation of NHA was sufficiently selective to give the final stable products observed in vivo and further indicate that NA is the precursor of nitrite and citrulline. Furthermore the same quantity of nitrite plus nitrate vs. citrulline were found at each potential, indicating that at each potential NHA is immediately oxidized into NA. The presence of the nitroso-adduct of NHA may be due either to the production at the electrode surface of H\(^+\) and nitrite which nitrosated NHA or to the degradation of NA indicating the generation of a nitrosating species between NA, citrulline and nitrite.

In a third series of experiments DPV was performed either with PRG5 or with Autolab. The DPV parameters were: sweeping rate 40 mV s\(^{-1}\), pulse height 100 mV and frequency 10 Hz. Using microelectrodes the different substances which were described for the NO pathway were studied in PBS (pH = 7.4) between +1.3 V in oxidation and −2 V in reduction vs Ag/AgCl. Peak potentials and detection limits are listed in Table 2. Microelectrodes

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**Table 1**

<table>
<thead>
<tr>
<th>Electrode potential (V)</th>
<th>Nitrite ((\mu)M)</th>
<th>Nitrate ((\mu)M)</th>
<th>Citrulline ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>63</td>
<td>230</td>
<td>310</td>
</tr>
<tr>
<td>0.8</td>
<td>83</td>
<td>202</td>
<td>330</td>
</tr>
<tr>
<td>1.2</td>
<td>43</td>
<td>232</td>
<td>390</td>
</tr>
<tr>
<td>1.6</td>
<td>36</td>
<td>285</td>
<td>290</td>
</tr>
<tr>
<td>2.0</td>
<td>55</td>
<td>287</td>
<td>220</td>
</tr>
</tbody>
</table>

* All values are the mean of three determinations. Statistical analysis showed that there was no significant difference between citrulline and the sum of nitrite and nitrate.

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Fig. 1. Cyclic voltammetry of hydroxy-arginine (NHA) in PBS. Two steps of the hydroxy-arginine (NHA) oxidation. Cyclic voltammetry at 40 mV s\(^{-1}\) in PBS with NHA (1 mg ml\(^{-1}\)).
detected NO both in oxidation and in reduction, while nitrite was only oxidized at the electrode surface. When NHA was oxidized at +1 V vs Ag/AgCl for 60 s, DPV in reduction showed the NA peak in reduction without NO peak (Fig. 3A). In vivo voltammetry was performed in living mice. After anesthesia with urethane (1 mg kg\(^{-1}\)) mice were held in a surgical head holder (D. Kopf, Roucaire, France). A hole was made in the skull. The dura was removed under microscopical observation and microelectrodes were inserted in the upper part of the frontal cortex using a micro-manipulator (Prior, Phymep, France). Auxiliary and reference electrodes were placed on the cortical surface. The animals were in a Faraday cage. Microelectrodes were implanted in the upper part (125 \(\mu\)M depth) of the frontal cortex. There was no peak in oxidation and a peak in reduction at \(-1.66\) V vs Ag/AgCl (Fig. 3B) with 185 nA height.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Peak potentials oxidation</th>
<th>Peak potentials reduction</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrulline</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>No</td>
<td>-0.36 and -1.02</td>
<td>1 mM</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>+1.21</td>
<td>-1.02</td>
<td>1 (\mu)M</td>
</tr>
<tr>
<td>Nitrite</td>
<td>+0.77</td>
<td>No</td>
<td>1 (\mu)M</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>+0.73</td>
<td>-1.37</td>
<td>1 (\mu)M</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>No</td>
<td>-1.58</td>
<td>10 (\mu)M</td>
</tr>
<tr>
<td>Superoxide</td>
<td>No</td>
<td>-0.79</td>
<td></td>
</tr>
<tr>
<td>NHA</td>
<td>+0.49 and +0.88</td>
<td>No</td>
<td>10 (\mu)M</td>
</tr>
<tr>
<td>X</td>
<td>No</td>
<td>-1.48</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>No</td>
<td>-1.66</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) All values were determined in PBS pH = 7.4 at 20°C using DPV with microelectrodes.
by cyclic voltametry.

...tion which represented the two steps previously described (Fig. 3D). In NHA solutions, DPV gave two peaks in oxidation which indicated the absence of NO and the same experiment was performed in reduction. In this case no or NO. In order to differentiate between NO and nitrite, the characteristics as NA. Adsorption experiments were performed in order to identify NA, based on the fact that a small quantity of NHA or NA can be stabilized on carbon microelectrode surfaces by adsorption as described for nitroso- or N-hydroxy-compounds [22]. An oxidation lasting a few seconds of NHA followed by DPV in reduction gives the NA signal without NO signal (Fig. 3A). Adsorption experiments which were performed in vivo allowed us to identify the structure of NA whereas in both reduction and oxidation NO was always undetectable. A potential of −2 V vs Ag/AgCl was fixed during 30 s at the electrode tip permitting adsorption and reduction of NA to NHA at the electrode tip. The subsequent DPV in oxidation gave three peaks in the nanoamp range (Fig. 3C). When this measurement was repeated again, the three peaks disappeared indicating that they were due to adsorption products. The first and third peak potentials are identical to NHA and the second peak at +0.7 V vs Ag/AgCl corresponded to nitrite or NO. In order to differentiate between NO and nitrite, the same experiment was performed in reduction. In this case no peaks appeared indicating the absence of NO and the consumption of NA during electrolysis which gave NHA (Fig. 3D). In NHA solutions, DPV gave two peaks in oxidation which represented the two steps previously described by cyclic voltametry.

DPV in NO-synthases incubation mixture was performed in a home-built open cell at ambient temperature (20°C). Ninety microliters of freshly prepared substrate received NO-synthase enzyme (10 µl), nicotinamide adenine dinucleotide phosphate (NADPH) 100 µM, 5-6-7-8-tetrahydrobiopterin 10µM, flavin-adenine dinucleotide (FAD) and flavin mono-nucleotide (FMN) 5µM, calmodulin 10 µg ml⁻¹ and CaCl₂ 500 µM. Measurements were made immediately for 10 min. The final concentration of NO-synthases was 10 µM. Rat brain recombinant NO-synthase expressed in a baculovirus system was 100 µM (Interchim, Montluçon, France). Rat brain NO-synthase was prepared from five fresh brains which were crushed in PBS, centrifuged, filtered and applied on a 2′5′ adenosine diphosphate (ADP)-agarose gel for 30 min. Then eluted NO-synthase by NADPH (5mM) was precipitated by ammonium sulfate (176 g l⁻¹), centrifuged (1000×g, 30 min, 4°C) and dissolved in PBS to adjust protein concentration to 100 µM. In these experimental conditions, no signal was observed in fresh or oxidized substrate, in NADPH, NADP solutions (100 µM) and for other cosubstrates at pH 7.4. No adsorption was observed in the conditions of the assay nor for PBS. When native and recombinant rat brain NO-synthases were tested, the peak of NA appeared immediately when the substrate was introduced with the enzyme. For 10 mM arginine the peak was a few hundred nanoamp (Fig. 4A,B). Performing the previously mentioned adsorption at −2 V vs Ag/AgCl, NHA and nitrite appeared in oxidation which confirms identification of NA. Furthermore, NA was quantified with DPV using nitroarginine, a stable compound, as a standard presenting two peaks at −1.4 and −1.7 V vs Ag/AgCl (see Ref. [11]). With this calibration, NA concentrations in the mouse brain or NO-synthases can be assessed. In these conditions, evaluation of Km and Vmax gave a Km of 4.3 and 1.8 mM with Vmax of 650 mM⁻¹ min⁻¹ mg⁻¹ and 95 mM⁻¹ min⁻¹ mg⁻¹ for the native enzyme and the recombinant one, respectively, (Fig. 4C,D). The NA concentrations at the microelectrode tip were ten to one hundred thousand times higher than that of nitrite or citrulline in a bulk solution which is due to the nature of the measurement with microelectrodes. NA was immediately trapped at the microelectrode surface and detected and then diffused giving nitrite and citrulline at a distance of a few microns from the microelectrode surface. However, the values for Km and Vmax obtained by classic determination methods were generally in the micromolar range and a few hundred nanomoles to 1 mM mg⁻¹ min⁻¹, respectively. Since micromolar arginine give nanomolar to micromolar NO, NO-synthases have been previously considered to be a suicide or poisoning enzyme. All compounds presumed to be produced by NO-synthase except citrulline were detected either in reduction or in oxidation by DPV with threshold of a few micromoles. Some of them such as NO, NHA and hydrogen peroxide gave a signal in each of the two domains. NA was the only one which could be detected with microelectrodes. Using amperometry for NO detection in NO-synthases two groups failed to detect NO without adding...
reducing species, probably due to interference with the cofactors. DPV confirmed that NO production, if present, was below 1 μM. This suggests that their production did not reach a significant level during normal activity of the enzyme. When measured by microelectrodes the ratio of NO and NA concentrations was at least 1000. This result suggests that NA is not a NO-donor molecule and that the breakdown of the NO group is not homolytic. Given the characteristics of the NA molecule, a heterolytic breakdown may give NO⁺ cation (nitrosonium). Nitrosonium is never present in a free form in solution due to a small half-life (milliseconds or microseconds). In an aqueous solution, a compound like nitrilo tetrafluoro-borate (NOBF₄), a nitrosonium donor, gives immediately the signal of NO in oxidation or in reduction. Since I only detected nitrite with microelectrodes, this signal may be due to NO₂ or nitrite generation during scission of the molecule in the presence of oxygen [4]. An alternative explanation is that only a very small proportion of NA gives NO, NO⁺ or NO₂ in active form, whereas its greater part, gives nitrite as an inactive form. In vitro studies have shown that nitric oxide is the only monoxide redox form which is able to activate soluble guanylyl cyclase [2]. NO₂ formed by reaction between NO and O₂ can also activate guanylyl cyclase.

NA could be transferred to its action site by amino-acid transport. Given its charge and its relatively low diffusion coefficient, its diffusion is limited to a few microns.

In conclusion voltametry and adsorption experiments were shown to be an appropriate tool to demonstrate the presence and structure of labile molecules like NA produced by NO-synthases from arginine and NHA. The products of degradation of NA are citrulline and nitrite. The formation of NA may enhance the selectivity of this pathway. The step between NA and nitrite and citrulline remains to be determined.

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