Advantages and limits of the electrochemical method using Nafion and Ni-porphyrin-coated microelectrode to monitor NO release from cultured vascular cells

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Electrochemical monitoring with a porphyrinic microsensor of *in situ* nitric oxide production from cultured cells offers numerous advantages but requires cautious analysis, repeated calibration and accurate localisation of the electrode. It also had some limitations. We describe here some characteristics of this method, the stability of the electrode response during experiments and its application to nitric oxide production by constitutive and inducible nitric oxide synthases. Real-time measurements of NO concentration allow the study of the kinetics of NO production. This is illustrated by the time-course of NO release from cultured human endothelial cells. How NO production by inducible nitric oxide synthase in cultured smooth muscle cells obtained from human internal mammary artery can be evaluated by Nafion- and Ni-porphyrin-coated electrode is also described. The results thus obtained are compared to the cumulated NO_2^- production evaluated by the Griess method.

Introduction

The measurement of nitric oxide (NO) is important for direct examination of the regulatory roles of this radical in various biological systems. Nitric oxide production indeed participates in the control of major cell functions including reactivity, proliferation and apoptosis. An inappropriate production or metabolism of this radical led to the development of various pathologies, including cardiovascular dysfunction, atherosclerosis, ischemia and neurovegetative diseases.

There has been an intensive search for accurate and specific detection methods to measure biological NO production (for review, see [1-3]). In a previous study of NO production from endothelial cells, we have compared three approaches, based respectively on the Griess reaction that quantifies NO release through its metabolites, nitrites and nitrates, on the transformation of haemoglobin into methaemoglobin by NO, and on the electrochemical NO detection with a porphyrinic microsensor [4]. These three methods were found to differ in terms of sensitivity and selectivity. The haemoglobin reaction and nitrate measurements suffer from a lack of specificity. Nitrite (and nitrate) determination by the Griess reaction was hardly suitable for

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kinetic studies but constitutes a rough indicator of cumulated NO production. In the present paper, we demonstrate further the capacity and limits of the electrochemical detection by differential pulse amperometry, that exhibits great sensitivity and specificity and allows instantaneous and non-disturbing measurements, but does not give direct access to continuous basal NO production, if any. We also describe that the activity of the inducible NO synthase isoform could however be evaluated with a porphyrinic microsensor.

Methods

Measurement of NO production

NO standards were prepared by serial dilutions of saturated NO solutions as described previously [5]. To produce a standard NO solution, deionised water solution was bubbled with argon for 20 minutes then with pure NO gas for 20 minutes and kept in a tight-capped vial until use. Dilutions of the saturated solution were made using deoxygenated water samples.

NO release was monitored with a NO-selective microprobe made of a carbon microfiber (8 µm diameter, approximately 1 mm length), coated with tetrakis (3-methoxy-4-hydroxyphenyl) nickel (II) porphyrin and Nafion films as described previously [5-7]. The last step of their preparation, Nafion coating, was layered on the day of experiments. Differential pulse amperometry was performed with a three-electrode potentiostatic system, Biopulse (Tacussel Radiometer, Lyon, France) or EMS 100 (Biologic, Claix, France) as previously described [8]. Potentials are expressed with reference to the aqueous standard calomel electrode (SCE). A large amplitude prepulse, from an initial potential of 0.40 V up to the working potential (0.78 ± 0.03 V), determined by differential pulse voltammetry for each electrode), was provided during 60 ms. The current I_1 was sampled during the last 6 ms of this prepulse and a pulse of 30 mV was applied for 60 ms during which was sampled the current I_2. Potential then returned back to the resting potential (400 mV). The difference between I_2 and I_1 constitutes the analytical signal. It was measured each s. All the apparatus was enclosed in a Faraday's chamber.

Internal calibration of the electrochemical sensor was performed for each experiment by adding NO standard solutions, as previously described [5,8]. The NO detection limit was in the 2-6 nM range. Under all experimental conditions described in this paper, it has been checked that none of the exogeneously added reactants interfere by itself with the electrode signal.

Measurement of nitrite production

Concentrations of nitrite accumulated in the cell medium were determined by the Griess reaction [9]. In acidic media, nitrites react with sulfanilic acid to produce a diazoïc compound, which leads to a purple azo dye with *N*-(-1-naphthyl)ethylenediamine. One hundred µl of the solution to be analysed was mixed with 100 µl of 0.1 M phosphate buffer (pH 7.4), 100 µl of 4N HCl and 100 µl of 0.04 N HCl solution containing 2 g/l sulfanilic acid, in 24 well plates. After stirring for 10 min, the colored dye was formed by adding 100 µl of a 1 g/l *N*-(-1-naphthyl)ethylenediamine dihydrochloride aqueous solution. The formation of the azo dye was measured 15 min later by spectrophotometry at 540 nm (Spectracount, Packard) and compared to calibration curve.

Cell cultures

Human umbilical vein endothelial cells were isolated from segments (10-30 cm-long) of human umbilical cord vein. They were cultured in medium 199 (40 %) and RPMI 1640 (40 %) containing foetal calf serum (20 %), penicillin/streptomycin (100 U/mL/100 µg/mL), L-glutamine (2 mM) and fungizone (2.5 µg/mL), as described previously [10,11]. The culture medium was renewed every other day. Cells in tissue culture dishes (35 mm diameter, Corning, New York, NY, USA) were studied at the first passage, when sub-confluent.Vascular smooth muscle cells were prepared from segments of human internal mammary artery obtained from patients undergoing coronary artery bypass graft surgery. They were isolated by using the explant method, according to Ross [12], as previously described [13]. Smooth muscle cells were cultured in Dulbecco's modified Eagle's medium containing 15 % foetal calf serum, penicillin (100 U/mL), streptomycin (100 µg/mL) and N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (8 mM). They were studied between passages 4 and 12, when sub-confluent. Cell viability was assessed by lactate dehydrogenase assay and trypan blue exclusion.

The NO-sensitive electrode was brought near the cell surface (at about 10 µm), by using a micromanipulator (Micromécanique, Évry, France) attached to an inverted microscope (Nikon, Japan), until both cells and tip of the electrode were located in the focal plane. Each culture dish was kept at 37 °C on a thermostated microscope platine (Micromécanique, Évry, France) and was stimulated only once. All the apparatus was enclosed in a Faraday's chamber.

Chemicals

Tetrakis (3-methoxy-4-hydroxyphenyl) nickel(II) porphyrin was from Interchim, Nafion and NO gas from Aldrich. Phosphate buffer solutions (PBS, pH 7.4) were prepared with 0.01 M phosphate salts from Gibco. N^{ω}-monomethyl-L-arginine (L-NMMA), bovine erythrocyte SOD, histamine, thrombin, LPS, TNFα and interleukin-1ß were purchased from Alexis Corp. All other chemicals were from Sigma Chemical Company.

Results and discussion

As it has been well established for electrochemical voltametric and amperometric methods using microelectrodes [14], NO detection with this porphyrinic microsensor can be considered as a non-destructive method. This can be

demonstrated theoretically or experimentally by comparison of the amount of NO oxidised during the experiment with its total amount in solution. If we consider the electrode area, the percentage of NO oxidized during an experiment can be calculated from the value of the diffusion limiting current in normal voltametry, as done for example for ferrocene that have a diffusion coefficient similar to that of NO [15]. It was clear from the Faraday law that NO oxidation current, integrated for the duration of the working potential, represents the oxidation of less than 1 % of the total NO amount in the sample. This implies that NO monitoring, as performed, does not modify the subtle equilibrium that controls cell NO production and metabolism, and the effector responses.

Variability of the electrode sensitivity during experiments

In agreement with previous data obtained with the same electrode system [5], NO oxidation gave a dose-dependent linear current response up to 150 nM, the highest concentration routinely tested (Fig. 1). The mean slope of calibration curves was 1.25 ± 0.17 pA/nM NO (n = 18, with a 95 % confidence interval of 0.90 to 1.60 pA/nM NO). The variability in slope and/or absolute current values corresponds respectively to the difference in sensitivity (depending on Nafion treatment) and in electrode area (reflecting the fiber length). Each electrode was calibrated before and during experiments, in the absence and presence of cells. It was observed that, under the experimental conditions described here, the electrode responses were not modified for at least 8 hours. Figure 1 illustrates the stability of NO oxidation signal at 3 different electrodes, at the beginning and at the end of serial experiments performed on the same day. Each electrode was used for a maximum of 2 days.

Nitric oxide production by constitutive eNOS: Kinetics of NO release in human endothelial cells, and significance

As the electrochemical approach allows real-time measurements of NO concentrations, NO stability can be measured from the time-dependent evolution of its oxidation signal. The rate of disappearance of NO standards in aerobic buffer increased with their concentrations, in accordance with previous observations [16,17]. Experimental half-life of a 1 µM NO solutions in aerated PBS buffer at 37 °C were 400 s while the apparent signal loss of a 10 nM NO solution was negligible during experiment.

NO stability in the presence of cultured endothelial cells was compared under two conditions, when produced by stimulation of the constitutive NOS and when authentic NO was added to the cell culture as internal standard. It is clear that the disappearance rate of NO released from endothelial cells stimulated by histamine or thrombin exceeded that of NO standards, even if they were added only a few minutes later at a concentration similar to that produced by stimulated cells (Fig. 2, traces a and c). This rapid decay underlines the short duration of eNOS activation by these agonists and rules out an exclusive NO consumption by spontaneous oxidation. At the low NO concentrations obtained, exchange with atmosphere is also negligible. The rapid reduction in NO levels can reflect either a simultaneous activation of degradation mechanisms, the reduction in the near membrane NO concentration due to diffusion far from the cell and the electrode tip, or both (Fig. 2). *In situ* degradation mechanism(s) should also be transient, as the stability of NO standards added 2-4 minutes after cell stimulation was not significantly altered. A transient production of superoxide anion can participate in NO consumption. However, addition of superoxide dismutase, which shortened its half-life, increased the maximal NO concentration but did not significantly prolong the NO signal. This suggests that NO dilution by diffusion [18] could also participate in the relatively rapid return to baseline of local NO concentration. Comparison of the transient NO release in thrombin- or histamine-stimulated cells, with the prolonged NO release observed in cells treated by thapsigargin, an inhibitor of Ca^{2+} uptake into internal stores (Fig. 2, traces b and d), also reveals that the characteristics of eNOS activation and/or NO degradation depends on the cell treatment. Kinetics of NO diffusion far from the cell membrane of thapsigargin-stimulated cells would be compatible with the experimentally observed long-lasting NO release only in case of sustained synthesis or in the absence of simultaneous stimulation of degradation mechanisms.

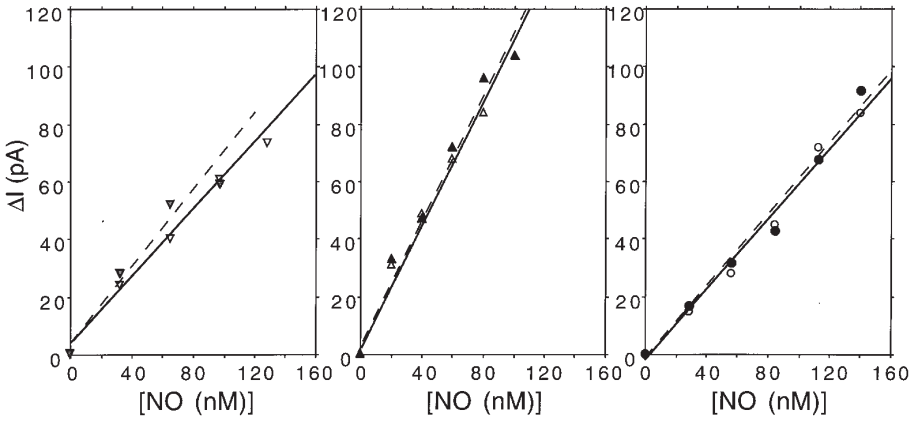

Figure 1. Stability of the electrode response. Comparison of calibration curves with authentic NO solutions obtained for 3 different electrodes, in buffered solution (filled symbols) and in presence of cultured vascular cells (open symbols).

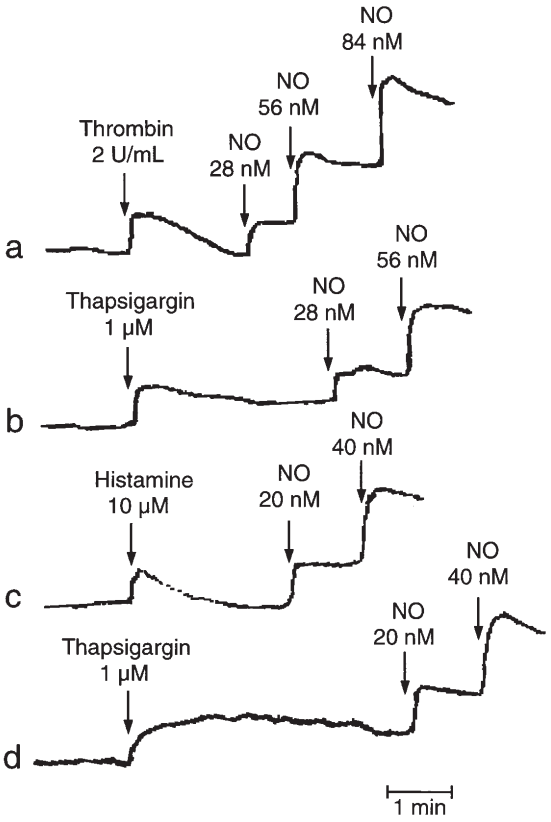

Figure 2. Comparison of the kinetics of NO release from cultured human endothelial cells stimulated by thrombin, thapsigargin or histamine, and fate of authentic NO solution added 2-4 min later in the cell medium as internal standard.

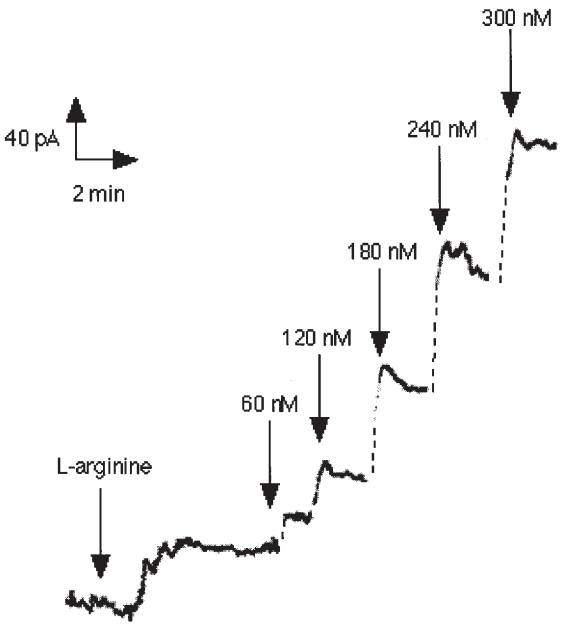

Figure 3. L-arginine-dependent stimulation of NO release in cultured smooth muscle cells from human internal mammary artery, after induction of the NOS II isoform by a 24 hour treatment with 10 ng/mL interleukin-1ß. Aliquots of authentic NO solution were then added as internal standard.

Nitric oxide production by inducible NOS in human smooth muscle cells

Nitric oxide production by inducible NOS has also been monitored by Nafion- and Ni-porphyrin-coated microelectrodes. Smooth muscle cells are capable of NO production after induction of Type II NOS. We have examined the release of NO from internal mammary artery smooth muscle cells (IMASMCs) pretreated by well-established inducers of NOS II. Confluent IMASMCs were treated for 24 h by lipopolysaccharides (30 µg/mL), Tumour Necrosis Factor-α (10 ng/mL) or interleukin-1ß (100 U/mL). Once induced, the type II NOS continuously produced NO. However, the amplitude of the basal current obtained by DNPA, which also includes the possible participation of other cell and medium components with same range of oxidation potential as well as capacitive current, cannot be considered as an exclusive indicator of NO production. Attempts to evaluate the basal NO production through the initial inhibition of the enzyme by L-NMMA, followed by the re-establishment of the enzyme activity by L-arginine addition were unsuccessful. This was likely due to the slow reversibility of L-NMMA-induced inhibition of NOS II [19]. We then used the increase in NO production associated to an enhanced L-arginine availability as index of NOS II activity. As observed in figure 3, addition of 0.5 mM L-arginine to interleukin-1ß-pretreated cells increased NO production within 2 minutes. In contrast with the transient agonist-induced NO release from endothelial NOS, NO production appeared to be stable for at least 6 minutes (the longer time studied). In untreated cells, the addition of 0.5 mM L-arginine increased NO production to a local concentration of 28 ± 5 nM (n = 11) (Fig. 4, upper panel). In LPS, TNF-α or interleukin-1ß-pretreated cells, this concentration was increased by 496 ± 107 % (n = 10), 294 ± 66 % (n = 3) and 339 ± 64 % (n = 8), respectively. Under these conditions, NO production by iNOS was in the 100-200 nM range, a value much higher than that obtained after eNOS stimulation. Addition of 1 mM L-NMMA, an inhibitor of NOS activity markedly suppressed the L-arginine-induced amperometric signal (Fig. 4, upper panel).

Under the same conditions, the nitrite concentration accumulated in the cell culture supernatant, that have been demonstrated to constitute a better index of NOS II activity in smooth muscle cells than nitrates [20,21] have been evaluated by the Griess method. After 24 hours, they reached 10 to 20 times higher concentrations than the instantaneous NO values given by the porphyrinic microsensor (Fig. 4, lower panel). In accordance with electrochemical determinations of NO release, nitrite production was enhanced in LPS, TNF-α or interleukin-1ß-pretreated cells, but not in the presence of L-NMMA, confirming that the L-arginine-dependent NO oxidation current indeed reflected the activation of the inducible NOS II.

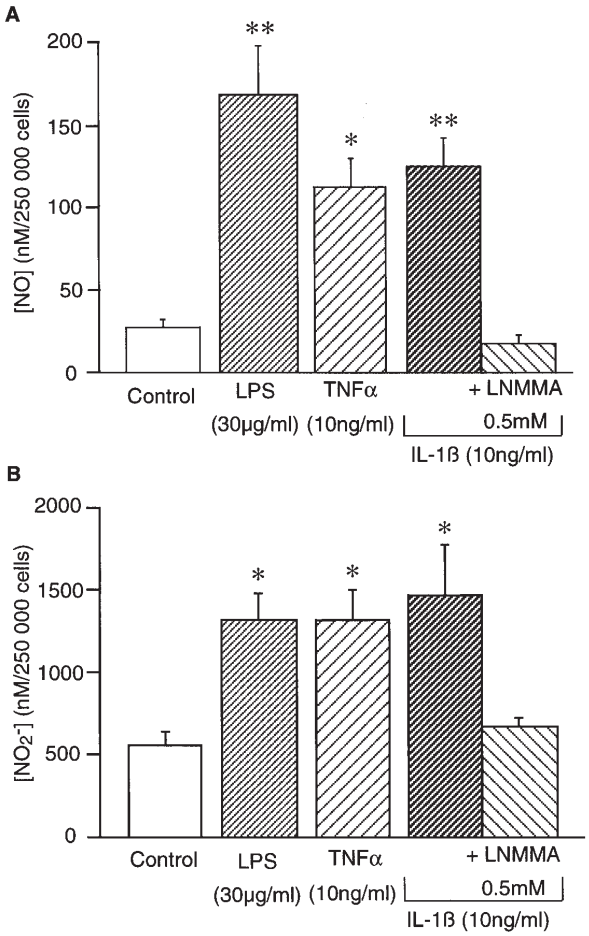

Figure 4. NO production induced by addition of 0.5 mM/L-arginine to cultured smooth muscle cells from human internal mammary artery, treated for 24 hours with bacterial lipopolysaccharides (LPS, 30 µg/mL), Tumor Necrosis Factor (TNFα, 10 ng/mL), or interleukin 1ß (IL-1ß, 10 ng/mL) with and without L-NMMA pretreatment. Nitric oxide release monitored at a Nafion and Ni-porphyrin-coated electrode (upper panel) was compared to the NO_2^- cumulated production for 24 hours determined by the Griess assay (lower panel).
Data are mean ±SEM from 3-10 independent experiments. * and **, P < 0.05 and 0.01 when compared to control values, respectively.

Conclusion

In our hands, monitoring NO production through its differential oxidation current at a Nafion and Ni-porphyrin-coated electrode offers several advantages but has however some limits.

The major advantages of following NO release from cultured cells through the electrochemical method described here are:

- A high specificity resulting from the association of various factors: the use of differential amperometry at a given potential allows to eliminate the interferences of other electroactive species oxidised or reduced at lower potentials, in contrast to direct amperometry. The presence of a Nafion layer opposes the access of anionic compounds, such as nitrites, to the electrode, without slowing down significantly its response time. Compared to NO detection, the selectivity ratios of Nafion and Ni-porphyrin electrodes for nitrite ions, xanthine, ascorbate, serotonine and L-arginine were 5600, 1100, 11000, 100 and 720, respectively [22]. This electrochemical approach also allows to extend the use of pharmacological tools to those exhibiting optical characteristics interfering with other methods of NO measurement.
- A short time response, in the ms range, allowing to monitor rapid changes in NO concentration and to determine real time-courses of production and degradation.
- A high sensitivity, in the nM NO range, due in part to the electrode size in the micron range, allowing to reach the NO concentrations locally produced by vascular cells. To limit the background fluctuations and improve the signal to noise ratio, already increased by the use of a differential method, we had to insert the system in a Faraday chamber. The whole apparatus was positioned on an anti-vibration table to keep as constant as possible the respective positions of electrode and cells and to select a heating microscope platinum devoid of electric signals.
- A limited influence on NO metabolism and its consequences, due to a minimal NO consumption. This induces a minimal and non-significant alteration of NO concentrations, allowing relevant studies of its release and metabolism, and more generally of the regulation of the generation of reactive oxygen species. This concerns for example the negative feedback regulatory role of NO on NO synthase activity [23], the modulation of superoxide-dependent oxidation and hydroxylation reactions [24] and the inhibition of catalase activity [25] by NO. All of these NO effects are obviously disturbed by indicators that bind NO with high affinity, compete with its physiological targets and disturb regulatory mechanisms. For example, this last point is illustrated by the much more prolonged NO production by polymorphonuclear neutrophiles stimulated by the chemotactic peptide fMLP evaluated by SOD-inhibitable oxidation of oxyhaemoglobin to methaemoglobin [26], than that observed at the NO electrode under similar conditions [27].

Other electrodes also proposed for NO monitoring do not share some of these characteristics. When compared to Clark-Type electrode, the Nafion and Ni-porphyrin-coated electrode has the advantages of a shorter response time, of a better selectivity due to the use of differential pulse amperometry, and of a better sensitivity. For example, Schmidt and Mayer have made several attempts to monitor NO release from cultured aortic endothelial cells by Clark-type electrode [28]. Independently of the incubation condition used (presence or absence of superoxide dismutase, L-arginine, bradykinin and Ca^{2+} ionophore) or of the experimental set up (detached cells, microbeads with attached cells or

cultured cell layer), they were not able to detect an NO signal. Similarly, the release of NO from 100 µM or 1 mM Sin-1 (a simultaneous O_2^- and NO donor that, given the O_2^- rapid disproportionation and high affinity for NO, behaves as an NO and ONOO- donor) could not be detected by a Clark-Type electrode in the absence of SOD [29] nor by a WPI NO sensor [30]. In contrast, using a Nafion and Ni-porphyrin-coated electrode, NO release from 100 µM Sin-1 could be studied even in the absence of SOD where it reached an equilibrium value in the 100 nM range [31].

The use of the method described here is however limited by:

- The possibility to follow the NO extracellular concentration only and not the in situ intracellular NO production. This limitation is however attenuated by the very high NO diffusion rate and permeability through cell membrane [32,33].
- The experimental variation in the apparent NO release due to the electrode position. The electrode has to be located as accurately as possible in a reproducible manner to minimise any apparent loss of signal associated with its distance from the cell surface.
- The difficulty to attribute the amplitude of the background oxidation current, reflecting the medium components oxidised near the working potential of the NO microelectrode, to NO concentration exclusively, unless the participation of all other electroactive species was evaluated. Although non-differential amperometric signals are considered as significant NO indicators in commercial NO electrochemical sensors, this interpretation should be carefully assessed in complex media containing various electroactive agents. This implies that only change in NO release, due to cell stimulation by agonists or acute changes in the enzyme substrate, activators or inhibitors can be safely interpreted as reflecting NOS activity. This is particularly important when the inducible NOS activity, which does not require additional stimulation, has to be analysed. Nevertheless, the present study demonstrates that changes in NO concentration induced by L-arginine addition reveal the activity of inducible NOS.

Electrochemical monitoring of NO is therefore a highly efficient tool that allows *in-situ*, direct and specific measurements of NO concentrations in aqueous medium. It can be considered as a nearly instantaneous and non-destructive method and thereby give access to physiologically relevant regulations controlling cell functions.

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