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Recording of NADPH Oxidation as a Means of Estimating NO Synthase Activity

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A novel approach to the measurement of NO synthase activity in brain tissue is described. In the NO synthase-catalyzed reaction, NADPH undergoes stoichiometric oxidation and, using a known specific inhibitor of this oxidation, NO synthase activity can be estimated by recording the oxidation rate. In the proposed approach, NADPH oxidation is recorded fluorimetrically, and the rate of this reaction in the presence of Nω-nitro-L-arginine is subtracted from its initial rate in the presence of L-arginine. This approach can be used to develop a simple, sensitive, and specific method for estimating NO synthase activity in brain structures of small animals.

Key Words: *nitric oxide; NO synthase; NADPH; brain*

The keen interest shown in the role played by nitric oxide (NO) in the central nervous system [3,10] has stimulated the development of methods for estimating the activity of the NO-synthesizing enzyme, NO synthase (NOS). Because the direct methods of NOS activity determination are complicated and not always reliable, the method resorted to most frequently to demonstrate the roles of NO and NOS *in vivo* involves inhibition of this enzyme by L-arginine analogs; indeed, it can be asserted with confidence that most of the data relating to NO functioning in the body have been obtained in this way. Unfortunately, such data are not open to a single interpretation, for it is not known which enzymes of arginine metabolism other than NOS can alter their activity in the presence of these inhibitors. Of the biochemical tests available for measuring NOS activity in brain tissue, the following four are most commonly employed: estimation of L-citrulline synthesis from radiolabeled L-arginine [4,9,11]; chemiluminescent determination of NO after its chemical reduction [2,12]; stimulation of soluble fibroblast guanylate cyclase as a measure of NO generation [5,7]; and spectrophotometric recording of oxyhemoglobin oxidation to methemoglobin in the presence of NO [7,8]. Generally, these tests are very time-consuming and/or insufficiently specific and for this reason are best suited for measuring the activity of purified NOS. EPR spectroscopy, with which NO generation in brain tissue can be fol-

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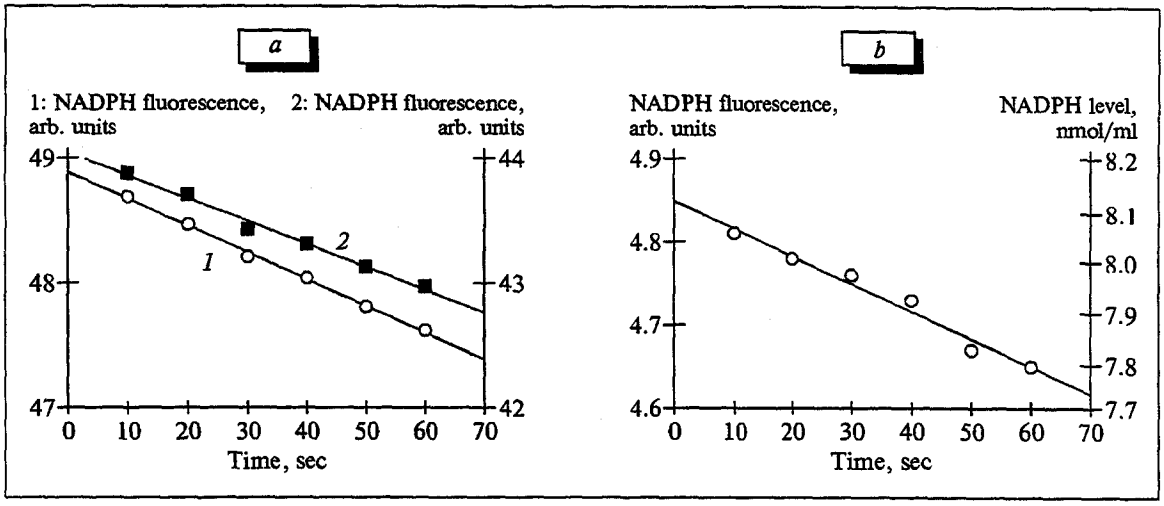

Fig. 1. Kinetics of NADPH oxidation by rat brain cytosol (10,000 g) in the presence of L-arginine (*a*, *1*) and Nw-nitro-L-arginine (NA) (*a*, *2*), and kinetics of NA-inhibited NADPH oxidation (*b*). The curve in *b* is the difference between oxidation rates *1* and *2* in *a*. Protein concentration = 1 mg/ml.

lowed [6,13], has also been used for estimating NOS activity [1], but this method requires a large quantity of biological material.

It follows, then, that a simple and reliable method of NOS activity estimation in brain tissue needs to be developed. The approach we have adopted rests on two well-established facts. First, NADPH undergoes stoichiometric oxidation in the NOS-catalyzed reaction of NO and L-citrulline production in the presence of molecular oxygen [2,9], and this oxidation can be registered by optical methods. Second, specific NOS inhibitors have been identified [10], of which Nω-nitro-L-arginine (NA) is particularly effective against NOS of the vertebrate brain [4] and inhibits its activity almost completely and irreversibly. Accordingly, we propose estimating NOS activity from the rate of NADPH oxidation blocked by a NOS inhibitor - NA in the case of brain tissue. Fluorimetric recording of the oxidation rate appears to be desirable for achieving the requisite sensitivity. NOS activity may thus be estimated by fluorimetric recording of the NADPH oxidation rate in two parallel samples, one with L-arginine and the other with the NOS inhibitor, followed by subtraction of the second value from the first; the difference will then yield the NADPH oxidation rate dependent upon the specific NOS inhibitor, i.e., an estimate of NOS activity.

MATERIALS AND METHODS

In this study we used whole brains and parts of brain from male Wistar rats (body weight 300-350 g) that had been maintained under standard conditions in the vivarium. The rats were decapitated and the brain was isolated, washed free of blood with chilled isotonic solution, and homogenized in the cold in a Potter homogenizer at 1500 rpm for 3 min in the isolation medium (50 mM Tris-HCl, 2 mM EDTA, 2 mM dithiothreitol, pH 7.4) in a 1:9 (I) or 1:5 (II) ratio. Homogenate I was centrifuged at 10,000 g for 30 min [4,11] and homogenate II at 100,000 g for 60 min at 0-4°C [5,8]. The cytosolic fractions obtained were either used at once as the source of NOS or kept frozen in liquid nitrogen until assay.

NADPH fluorescence was recorded with a Hitachi F-3000 spectrophotometer in quartz cuvettes having an optical path length of 1 cm at 37°C (excitation maximum 340 nm, emission maximum 460 nm). In the routine tests, the incubation medium contained 25 mM Tris-HCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM NADPH, 1 mM L-arginine or 1 mM NOS inhibitor (usually NA), and cytosol (0.2-1 mg protein/ml), pH 7.4. The mixture was preincubated without the cytosol for 5 min at 37°C to stabilize NADPH fluorescence, after which the cytosol was added and the reaction kinetics recorded for 1 min.

All the reagents used were from Sigma, with the exception of $MgCl_2$ (chemically pure; Reakhim, Russia).

RESULTS

As shown in Fig. 1, the oxidation rate was linear during the first minute of incubation. Adding L-arginine to the sample did not, as a rule, have a substantial effect on the NADPH oxidation rate because the L-arginine concentration in brain tissue is high (about 0.1 mM). Nonetheless, we

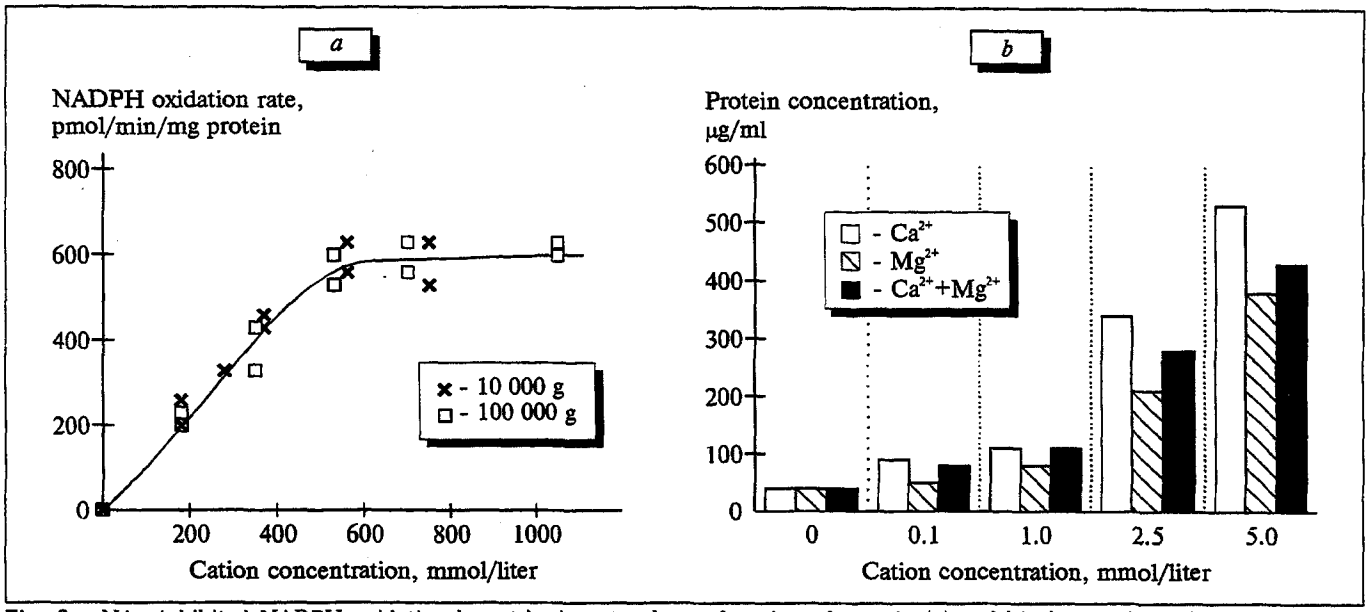

Fig. 2. NA–inhibited NADPH oxidation by rat brain cytosol as a function of protein (*a*) and bivalent cation (*b*) concentrations. *b*) $Ca^{2+}+Mg^{2+}$ were taken in a 1:1 ratio and the figures give their total concentrations.

deemed it necessary to add substrate amounts of L-arginine to the samples in order to standardize the method [8]. NADPH concentrations used in different methods of NOS activity estimation usually range from 0.1 to 2 mM; as we found in a preliminary experiment, raising the NADPH concentration above 0.1 mM will not alter the oxidation rate.

NA was found to inhibit NADPH oxidation to the maximum (to block it completely according to the data reported by Dwyer *et al.* [4]) in a concentration of 1 mM. N^G-monomethyl-L-arginine was less inhibitory. Thus, while the rate of NADPH oxidation by cerebellar (100,000 g) cytosol in the sample with L-arginine was 680 pmol NADPH/min/mg protein, the NADPH oxidation rate in the sample with 1 mM NA was 230 and in the sample with N^G-monomethyl-L-arginine 520 pmol NADPH/min/mg protein. These results are consistent with the view that NOS of the mammalian brain is inhibited most effectively and completely by NA [4]. The share of NA-inhibited NADPH-oxidase activity in the total NADPH-oxidase activity in the presence of L-arginine varies with the species of animal and from one brain region to another, but is generally no less than 15% and no more than 40%, the usual range being 20-30%. Since the inhibitory efficacy of NA greatly increases as a result of its preincubation with the enzyme [4], the effective NA concentration may be lowered by introducing a stage of NA preincubation with NOS.

Figure 2, *a* shows a curve of NA-inhibited NADPH oxidation rate plotted against protein concentration. It can be seen that the method of cytosol production (i.e., centrifugation speed) had little effect on NOS activity and that the NADPH oxidation rate was linear at protein concentrations under 0.6 mg/ml.

The dependence of NOS activity upon Ca^{2+} has been thoroughly examined with purified preparations of the enzyme [7,12]. However, the Ca^{2+} concentrations used in the different procedures differed by several orders of magnitude [2,4,5,8,9,12]. Moreover, in some cases Ca^{2+} is not added to the media at all in the belief that it is present in sufficiently high concentrations in the reagents or biological material used, especially if the latter is the brain cytosolic fraction rather than purified enzyme. Although many workers add Mg^{2+}, the effect of this cation on NOS activity remains virtually unexplored. [5,7,8,12]. It follows from Fig. 2, *b* that the NA-inhibited NADPH oxidation rate does depend on both Ca^{2+} and Mg^{2+} and that only millimolar concentrations of these cations are required for NA to display its maximal activity.

NOS activity, as estimated by NADPH oxidation, amounts to hundreds of pmol per min per mg protein; in the cerebral cortex and cerebellum of intact male Wistar rats (body weight 300-350 g), for example, it was found to be 648±64 and 772±110 pmol/min/mg protein, respectively (data averaged for 5 animals). The activity of constitutive NOS has been variously reported to range from several pmol to hundreds of nmol NO per min per mg protein depending on the degree of enzyme purification and the method of estimating activity [1,2,6-8,11]; NOS activity values measured

in the present study by NADPH oxidation lie within the range of values most commonly cited for NOS activity in brain cytosol.

In summary, the rate of NA-inhibited NADPH oxidation can be reliably and reproducibly measured in brain tissue specimens. Since NA is considered to be a specific inhibitor of mammalian brain NOS [4], and since NADPH oxidation proceeds stoichiometrically with NO formation in a NOS-catalyzed reaction [9], it may be stated that the NA-inhibited NADPH oxidation rate reflects NOS activity in the brain tissue. The proposed approach can also be used for estimating NOS activity in other tissues provided a suitable enzyme inhibitor is used.

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