BIOPHYSICS AND BIOCHEMISTRY

Determination of NO-Synthase Activity in the Brain: A New Method

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The method is based on the well-known reaction of nitric oxide with diethyldithiocarbamate and Fe(II), yielding a paramagnetic mononitrosyl complex, measurable by EPR spectroscopy.

Key Words: *NO-synthase; method; rat brain*

NO-synthase is an enzyme synthesizing nitric oxide (NO) from L-arginine in various tissues. Due to the the key role of NO in the transduction mechanisms in blood vessels, platelets, and in the nervous system, and its effector role in immune reactions, NO-synthase represents one of the most important regulatory systems and is now being extensively studied [6]. Apart from a constitutional form of the enzyme, a potent induction of the enzyme in response to various agents has been found in many tissues. At present, there is no direct evidence of the induction of NO-synthase in the brain, where the enzyme participates in the regulation of the brain circulation [8] and couples the activation of the postsynaptic N-methyl-D-aspartate receptors with functional changes in neurons and glial cells [6] by catalyzing the synthesis of equimolar amounts of NO and citrulline from L-arginine [1]. Altered expression of mRNA of NO-synthase has been found during the development of some disorders in the central nervous system [4] and in stress [2].

NO-synthase activity is routinely determined by measuring the conversion of L-[2,3-^{3}H]-arginine into L-[2,3-^{3}H]-citrulline by HPLC [7]. This method is extremely complicated, time-consuming, and expensive. A simpler method is based on a spectrophotometric assay of methemoglobin Ch, a product of oxidation of oxyhemoglobin by NO in the presence of sodium nitroprusside [3]. This method may be used exclusively for purified enzyme, not for crude homogenates or partially purified preparations, which contain a certain amount of hemoglobin and other methemoglobin-producing compounds. However, the assay of NO-synthase in dense homogenates is of particular interest, since these preparations contain effectors modulating the activity of the enzyme *in vivo* and thereby reflect the actual activity of NO synthesis in a particular tissue under certain conditions.

The aim of the present study was to develop a method for functional investigation of NO-synthase activity in the brain tissue. The method developed by us is based on the well-known reaction of nitric oxide with diethildithiocarbamate (DETC) and Fe(II), yielding a paramagnetic

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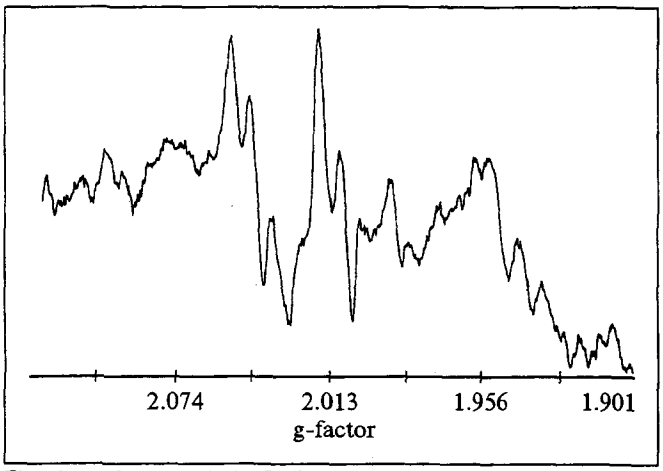

Fig. 1. Characteristic EPR spectrum of brain homogenate sample, containing a DETC–Fe–NO complex.

mononitrosyl complex, which may be measured by EPR spectroscopy [5].

MATERIALS AND METHODS

White rats were decapitated, the brain was promptly removed and placed in cool Tris-HCl buffer (pH 7.4). The brain was washed and 30-50% homogenates were prepared in a 50 mM HEPES buffer (pH 7.4) containing 2 mM $CaCl_2$. DETC was added to the homogenates in a final concentration of 1 mg/ml. It was shown that there is no need to supplement dense homogenates with Fe(II), since its concentration is high enough and does not limit the formation of the DETC-Fe-NO complexes. The homogenate samples were supplemented with substrates 1 mM L-arginine and 1 mM NADPH and incubated at 37°C. After the incubation was completed, a 0.7 ml aliquot was placed in a 30-mm-long Teflon tube with an internal diameter of 6 mm, which was then sealed at both ends and frozen in liquid nitrogen. The frozen sample was removed from the tube and placed in a Dewar flask preliminarily cooled with liquid nitrogen for EPR spectroscopy. The EPR spectra were recorded with a Bruker ESP300 spectrophotometer under the following conditions: 400 G field set, 100 kHz frequency modulation, 5 G amplitude modulation, 80 sec scan time, and 0.5 sec time constant. For estimation of the absolute concentration of DETC-Fe-NO complexes a reference complex of a known concentration and with the same geometrical parameters was prepared. The EPR spectrum of the reference complex was recorded and the absolute concentration (or amount) of NO in the tested sample was estimated by comparing the standard and tested EPR spectra.

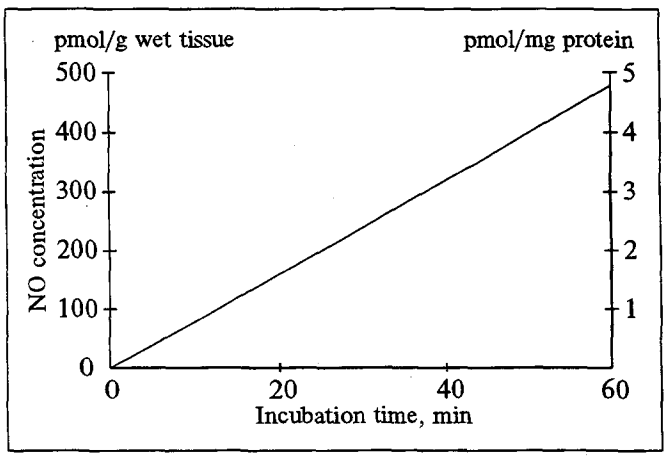

Fig. 2. Characteristic time course of accumulation of NO (as a constituent of a DETC–Fe–NO complex) in 30% brain homogenate.

RESULTS

In biological samples treated with DETC the EPR signals may arise from complexes of DETC with copper and triple DETC-Fe-NO complexes [5]. The EPR signal of a DETC-Fe-NO complex is localized at a mean g-factor of 2.03 and represents a typical three-component signal (Fig. 1). The EPR spectra of DETC-copper and DETC-Fe-NO complexes partially overlap, but in the particular case of brain homogenates the contribution of the EPR signal of DETC-copper complexes to the high-field component of the DETC-Fe-NO EPR signal is negligible. This makes it possible to evaluate the amount of a DETC-Fe-NO complex and thereby the concentration of NO from the intensity of the high-field component of the signal of this complex. Figure 2 shows a typical time course of the accumulation of a DETC-Fe-NO complex in a 30% homogenate. As is seen from the figure, the rate of accumulation of the DETC-Fe-NO complex is linear at least during the first 40 min of incubation. The enzyme activity is 87 pmol/min/g wet tissue or 0.9 pmol/min/mg protein, which is consistent with published data [7]. NO-synthase activity in homogenates of different brain areas of outbred and Wistar rats of different ages and both sexes ran-ged from 25 to 300 pmol NO/min/g wet tissue (0.25-3.0 pmol NO/min/mg protein). The minimal measurable amount of NO in the sample is 0.2 nmol.

The fact that the method proposed here allows for specific measurement of NO-synthase activity is additionally confirmed by the following experimental data: 1) there was no accumulation of DETC-Fe-NO complexes when the animal was killed with a superhigh frequency agent, which denatures proteins and, consequently, inactivates all enzymes; 2)

the accumulation of the DETC-Fe-NO complex was completely blocked by 2 mM ethylene glycol tetraacetate, a specific chelator of Ca^{2+} ions, which is an obligatory component of the NO-synthase reaction; 3) the accumulation of the DETC complex was inhibited by the specific inhibitors of NO-synthase N-methyl-L-arginine (by 70% at a concentration of 1 mM) and nitro-L-arginine (by 60% at a concentration of 1 mM).

Thus, the method proposed here may be used for correct determination of NO-synthase activity in dense brain homogenates. The method is also applicable for enzyme preparations of various purity (in these cases auxiliary problems concerning the incubation conditions and addition of Fe(II) should be experimentally solved), but in the case of dense homogenates and tissue samples, where the level of effectors regulating NO-synthase activity is maximally preserved, this method offers the greatest advantages.

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Antenatal Effects of Ethanol and Limontar on Lipid Peroxidation and the Antioxidant Defense System in the Brain and Liver of Rat Progeny

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In contrast to short-term exposure, prolonged exposure to ethanol in the antenatal period is found to inhibit lipid peroxidation in the brain and liver of rats. Activation of the system of antioxidant defense in the brain and liver is observed after both short- and long-term exposure to ethanol. After short-term ethanol exposure, limontar normalizes lipid peroxidation.

Key Words: *antenatal effect of ethanol; lipid peroxidation; enzymatic system of antioxidant defense; brain; liver*

Lipid peroxidation (LPO) activated by ethanol impairs biological membranes and their permeability [10,12]. The enzymatic system of antioxidant (AO) defense also responds to ethanol administration [5,13]. These processes are still to be researched in newborns exposed to ethanol before birth. Such studies are necessary in order to find ways of protecting the fetus from the toxic effect of ethanol.

Previously we investigated LPO and the activity of AO defense enzymes in the brain and liver of rat fetuses and newborns after a short-term exposure to ethanol [1].

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