Activity of NO-Synthase and Radical Formation in Rat Brain Compartments: Age Dependence

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 120, № 7, pp. 54-56, July, 1995
Original article submitted July 21, 1994

The relationship between age and the activity of NO-synthase and generation of free radicals in different compartments of the brain was studied in male Wistar rats. NO-synthase activity was highest in the cerebellum and lower in the cerebral cortex. It increased with age in the cerebellum and remained unchanged in the cortex, being virtually the same in the right and left hemispheres. Radical generation was much higher in the cerebellum than in the cortex and, as a rule, was age-dependent. The ratio of NO-synthase activity to radical generation was age-dependent: a tendency toward a positive linear correlation was observed in young animals, no correlation could be traced in adults, and a negative one was observed in old rats.

Key Words: *nitrogen oxide; NO-synthase; free radicals; chemiluminescence; brain*

The role of nitric oxide (NO) in the functioning of the central nervous system has attracted growing interest in recent years [14]. The source of NO in the brain is NO-synthase (NOS) activity. Constitutive (in the neurons) and inducible (in glial cells) forms of NOS have been detected in brain tissue [9]. The relationship between NOS activity and age has not been studied up to the present time, nor has the regional distribution of the enzyme in the brain been assessed by directly measuring its activity. The regional distribution of the enzyme synthesizing the vessel-relaxing factor (NO) in the rat brain has been studied by measuring guanylate cyclase activity, which cannot be considered as a reliable indicator of NOS activity [3].

The reaction of L-arginine oxidation catalyzed by NOS includes, along with citrullin formation, the generation of at least two products of a free-radical nature: NO and superoxide [11], the interaction between these radicals leading to the formation of highly reactive compounds which are believed to be important factors in NO neurotoxicity [5,6,8]. A close relationship is hypothesized between NOS activity and the generation of reactive oxygen species in brain tissue. Measurement of chemiluminescence (CL) is a well-known method for assessing free-radical generation, despite the fact that different reactions may be the source of CL [13]. Analysis of H_2O_2-induced CL helps assess the potential generation of free radicals by tissue [15].

Our task in this study was to investigate the relationship between age and the activity of NOS and free-radical generation in some compartments of the brain of Wistar rats.

MATERIALS AND METHODS

Thirty-nine male Wistar rats kept in a vivarium with unlimited access to water and food were examined, 6 of these being young (aged 2.5

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0007-4888/95/0007-0700$12.50

months), 15 adult (5.5 months), and 18 old (23 months) animals.

The rats were decapitated and the brain was rapidly isolated, washed in ice-cold isotonic NaCl solution, and frozen in liquid nitrogen. The cerebellum, left and right cerebral cortex, and subcortical structures were isolated from the frozen brain and homogenized with 2 volumes of a buffer containing 50 mM HEPES, pH 7.4, and 1 mM $CaCl_2$. Samples of these homogenates were used to measure NOS activity, and the remaining portion was centrifuged at 3000 g for 10 min; the supernatants were used to measure CL.

NOS activity was measured by recording mononitrosyl complexes of NO with diethyldithiocarbamate and bivalent iron [1]. CL (maximum emission) was recorded after H_2O_2 induction [12] using a KhLM-3M chemiluminometer (Russia) at 20°C in glass cuvettes. Luminol (50 μl of a 0.34 mM solution) and 20 μl of 400-times-diluted supernatant was added to 1 ml of buffer, pH, 7.4, containing 20 mM KH_2PO_4 and 115 mM KCl. The reaction was triggered by adding H_2O_2 in a final concentration of 0.48 mM.

Sigma chemicals were used in the study.

Statistical analysis was carried out using STATGRAPHICS software.

RESULTS

Figure 1 presents the results of measuring NOS activity in various compartments in the brain of rats of different age. As a rule, NOS activity in the cerebellum was higher in adult rats than in pups and was virtually the same in old animals as in adults. In other structures this activity was not age-dependent. The highest activity was observed in the cerebellum and subcortical structures, but differences between NOS activity in the cerebellum and other structures were not as great as those reported by some authors [3], who assessed this parameter by indirect methods. No obvious asymmetry was observed in the distribution of NOS activity in the cerebral cortex, although the asymmetry coefficient (ratio of NOS activity in the left and right hemispheres) in young rats (1.22±0.07) differed from that in adult (0.95±0.08, $p<0.05$) and old (0.99±0.06, $p<0.06$) animals. ANOVA analysis of variance revealed a reliable age dependence of NOS activity only in the cerebellum ($F=3.74$, $p<0.04$).

The positive linear correlations of NOS activity in various portions of the brain revealed in old rats were indicative of a concordant pattern of regional changes of NOS activity in this group: cerebellum - left hemispheric cortex, cerebellum - right hemispheric cortex, cerebellum - subcortical structures, left - right hemispheres, left hemisphere

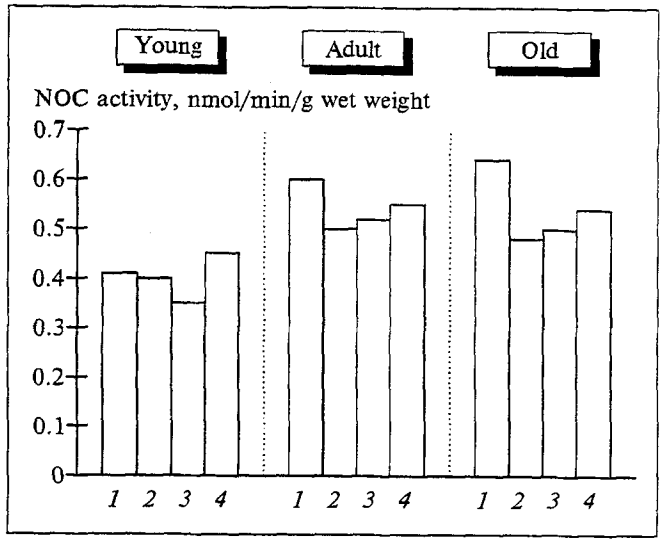

Fig. 1. NOS activity in brain compartments of rats of different age. Here and in Fig. 2: *1*) cerebellum; *2*) left hemisphere; *3*) right hemisphere; *4*) subcortical structures. Differences are reliable by the nonparametric Wilcoxon–Mann–Whitney test. Between brain compartments within one group: adult rats, *1–3* ($p<0.03$) and *1–2* ($p<0.02$); old rats, *1–3*, *1–2*, *3–4*, and *2–4* ($p<0.01$). Between the same brain compartments in different groups: *1*) adult–young ($p<0.005$), old–young ($p<0.01$); *3*) old–adult ($p<0.03$), adult–young ($p<0.002$).

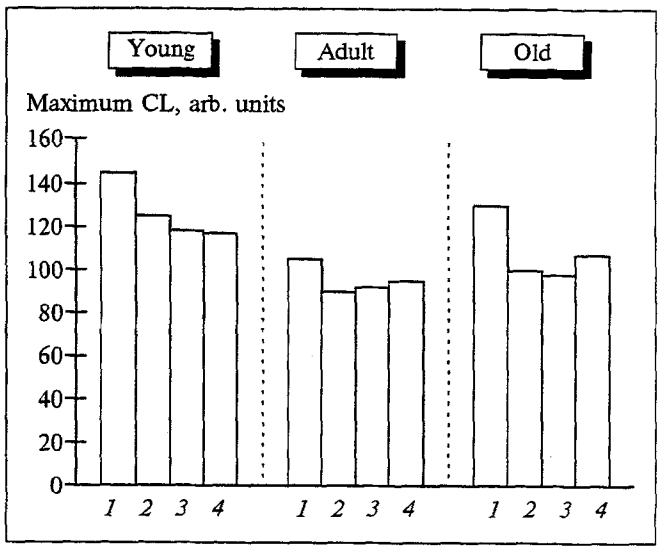

Fig. 2. Intensity of H_2O_2–induced luminol–dependent CL in the soluble fraction of the brain in rats of different age. Differences reliable by the nonparametric Wilcoxon–Mann–Whitney test. Between brain compartments within one group: young rats, *1–4* ($p<0.03$); adult rats, *1–3*, *1–2* ($p<0.001$), *1–4* ($p<0.01$), *2–4* ($p<0.005$), *3–4* ($p<0.02$); old rats, *1–3* ($p<0.002$), *1–2* ($p<0.004$), *1–4* ($p<0.02$). Between the same brain compartments in different groups: *1*) old–adult ($p<0.02$), adult–young ($p<0.001$); *3*) old–adult ($p<0.04$), adult–young ($p<0.002$), old–young ($p<0.07$); *2*) adult–young ($p<0.001$), old–young ($p<0.02$); *4*) old–adult ($p<0.04$), adult–young ($p<0.002$).

- subcortical structures, right hemisphere - subcortical structures (r=0.73-0.93, p<0.001-0.0001).

Figure 2 presents data on CL intensity in various compartments of the brain. As a rule, CL was appreciably higher in the cerebellum than in the cortex and subcortical structures. At first CL diminished with age (pups → adults) but then it increased (adults → old rats). No reliable correlations between CL values in various compartments of the brain were detected in the studied groups of animals.

Some reports suggest a close relationship between the generation of free radicals and NOS activity: *in vitro* generation of superoxide radical [11] and hydrogen peroxide [5] by the brain NOS; *in vitro* NO reaction with superoxide and organic peroxide radicals [10]; thermodynamic possibility of generation of a hydroxyl radical from NO [6]; *in vitro* initiation of lipid peroxidation in low-density lipoproteins by NO and superoxide [2]; N-methyl-D-aspartate receptor-induced increase of the generation of superoxide [7] and hydroxyl [4] radicals *in vivo*. These data provide a basis for assuming that NOS plays an important role in the formation of the free-radical status of brain tissue and that there is a positive correlation between NOS activity and free-radical generation. Nonetheless, in no case did we detect a positive correlation between these parameters. Moreover, in old rats negative correlations were observed (both as a tendency and statistically reliable) between NOS and radical generation in the cerebellum (r=-0.41, p<0.08), left hemisphere (r=-0.52, p<0.03), right hemisphere (r=-0.51, p<0.03), and subcortical structures (r=-0.33, p<0.2). In young rats statistically negligible tendencies toward positive correlations between NOS and radical generation were seen (r=0.27 to 0.69, p<0.4 to 0.13 in various compartments of the brain; the lack of statistical reliability may be due to the small number of animals per group). In adult rats neither statistically reliable correlations between NOS and chemiluminescence nor evident tendencies were detected.

Hence, the relationship between NOS activity and free-radical generation in brain tissue is much more intricate than might be hypothesized from *in vitro* data, and it appears to depend on the age of the animal.

The authors are grateful to A. I. Tsapin for his assistance in recording the electron paramagnetic resonance spectra.

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