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SHORT COMMUNICATION

NADPH diaphorase reactive neurons in temporal lobe cortex of patients with intractable epilepsy and hippocampal sclerosis

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Summary Several studies have demonstrated a controversial involvement of NO in epileptogenesis.

The aim of this study is to compare the NADPH diaphorase (NADPH-d) reactivity in the temporal cortex between surgical specimens of patients with intractable epilepsy and hippocampal sclerosis and autopsy controls.

Brain samples of patients and postmortem controls were stained with the NADPH-d technique.

Sprouting and larger areas of NADPH-d reactive neurons were found in the temporal cortex of epileptic patients.

Introduction

Nitric oxide (NO) is a short-lived free radical with diverse functions as a biological messenger molecule, and is implicated in numerous aspects of the physiology and pathology of CNS (Dawson and Dawson, 1994; Chung et al., 2005).

NO is synthesized by different isoforms of NO synthase (NOS): endothelial (eNOS), neuronal (nNOS), and the inducible isoform (iNOS). Neurons containing nNOS can be accurately localized using nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemical technique (Vincent and Kimura, 1992).

Several experimental studies have demonstrated the involvement of NO system in epileptogenesis (Ferraro et al., 1999; Murashima et al., 2000), however, the functional meaning remains controversial and NO can act as proconvulsant (Osonoe et al., 1994) or anticonvulsant, (Kirkby et al., 1996; Kato et al., 2005; Noh et al., 2006), depending on the experimental model used.

There are only two reports about changes in NO levels or NOS expression in human epileptic brain. A NOS up-

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regulation in the temporal cortex of patients with a long seizure history (González-Hernández et al., 2000), and a decrease of hippocampal nNOS with an increase of nNOS fibers throughout the fascia dentata (Leite et al., 2002) was described.

In this study, we compared the morphology and size area of NADPH-d reactivity of neurons found in the temporal lobe cortex (TLC) of epileptic patients with hippocampal sclerosis, who underwent anterior temporal lobectomy for intractable seizures versus postmortem normal controls.

Methods

Patients and samples

Five males and two females epileptic patients who underwent anterior temporal lobectomy for refractory TLE with unilateral hippocampal sclerosis detected by MRI (magnetic resonance image) were included. The mean age was 35 ± 11.6 years, the epilepsy time duration 26.6 ± 10.3 years, and the age of epilepsy onset was 9.1 ± 6.4. A right temporal lobe resection was conducted in five patients and a left one in two patients. Tissue blocks of $7\,mm \times 5\,mm \times 5\,mm$ were obtained from lateral and basal cortex areas from anterior temporal lobe extirpated during surgery. The temporal lobe showed neither MRI evidences of damage nor macroscopic or microscopic pathological findings using conventional techniques.

All patients underwent a thorough clinical, electrophysiological and imaging evaluation prior to surgery. All the samples were studied by a neuropathologist to confirm the diagnosis.

Postmortem control sample brains ($n = 5$) of four males and one female, mean age 65.4 ± 9.5 without pathological evidences were obtained from archival material of autopsies. The time lapse between death and autopsy never exceeded 6 h.

The studies were conducted with the approval of the Ethics Committee of the Ramos Mejía Hospital and all the subjects received informed consent.

Tissue processing

Tissue blocks were fixed in a solution containing 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h at 4 °C. and were washed in 0.1 M sodium phosphate buffer (PB) (pH 7.4) for one day at 4 °C. Coronal sections (thickness: 40 μm) were incubated in a solution containing ß-NADPH (1 mg/ml) and nitroblue tetrazolium (0.2 mg/ml) (Sigma Chemical Company, St Louis, MO), in 0.1 M PB, pH 7.4, at 37 °C for 1 h. All sections were processed at the same time and negative controls were incubated in the same solution but omitting NADPH.

Image analysis

The images were acquired by a SONY Power Had 3CCD color video camera system from a Nikon Optiphot II microscope attached to a KONTRON KS 400 image analyzer. Images were digitized with a resolution of 768×494 pixels (1 pixel = 1.70 μm at 20×). Neuronal reactivity areas were determined and average reactive size and standard deviation (S.D.) were calculated. Student's *t*-test was determined, using the SPSS for Windows.

Results

In both groups of sections NADPH-d reactive neurons with nonpyramidal morphology were localized preferentially in layers II, IV, V, VI and in the immediately subjacent white matter of the temporal lobe cortex.

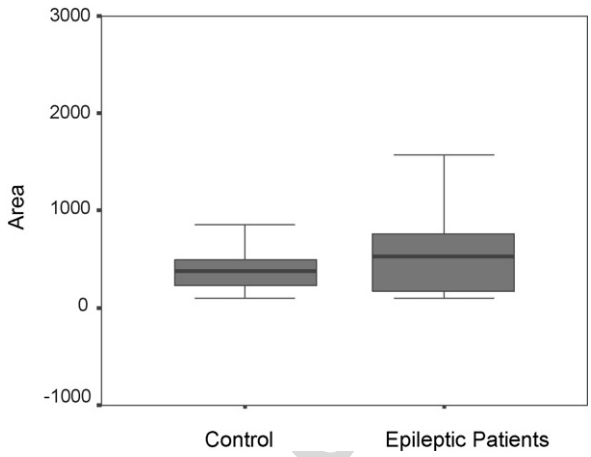

Fig. 1 Box plot of the area of NADPH-d reactive neurons. Area: pixel^2; Controls, $n = 5$, epileptic patients $n = 7$, $p < 0.001$.

The reactive neuronal area in the temporal lobe cortex (TLC) of epileptic patients compared to the control group was 555 ± 388 versus 393 ± 190 pixel^2, respectively ($p < 0.001$) (Fig. 1).

Epileptic NADPH-d neurons showed multipolar morphology and an increase of the dendritic arborization of NADPH-d processes, characterized by a higher ramification, longer reactive dendrites, higher number of dendritic spines, and in consequence larger areas ($p < 0.001$) (Fig. 2A—D). NADPH-d neurons in controls were bipolar fusiform and showed thinner dendrites with less dendritic spines (Fig. 2E—H).

Discussion

NADPH-d neurons were originally described as ''the solitary active cells in the cerebral cortex''. These cells also contain NPY, somatostatin and GABA and have been considered inhibitory interneurons (Vincent and Kimura, 1992).

NO seems to be generated as a consequence of the kindling phenomena (hyperexcitability) in experimental models of epilepsy. A remarkable increase of extracellular levels of NO metabolites, an increase of NOS immunoreactivity and a nNOS upregulation was found in cerebral brain regions affected by epileptic discharges (Kato et al., 2005; Murashima et al., 2000; Itoh et al., 2004), including contralateral temporal lobe structures (Yasuda et al., 2001).

In human epileptic patients who underwent neurosurgery, one study showed an increase in the number and the staining intensity in cortical neurons, with more intensive NOS reactivity in patients with a high number of seizures over a longer period (González-Hernández et al., 2000). All patients in the present study were epilepsy refractory patients, with a long seizures history.

The dendritic arborization of NADPH-d reactivity, accompanied by an important quantity of dendritic spines, could represent a plastic change in response to the chronic discharges. The found morphological changes could represent a sprouting phenomenon of NADPH-d reactive neurons. A previous observation of sprouting of nNOS was reported in the fascia dentata's fibers of human hippocampus with hippocampal sclerosis (Leite et al., 2002).

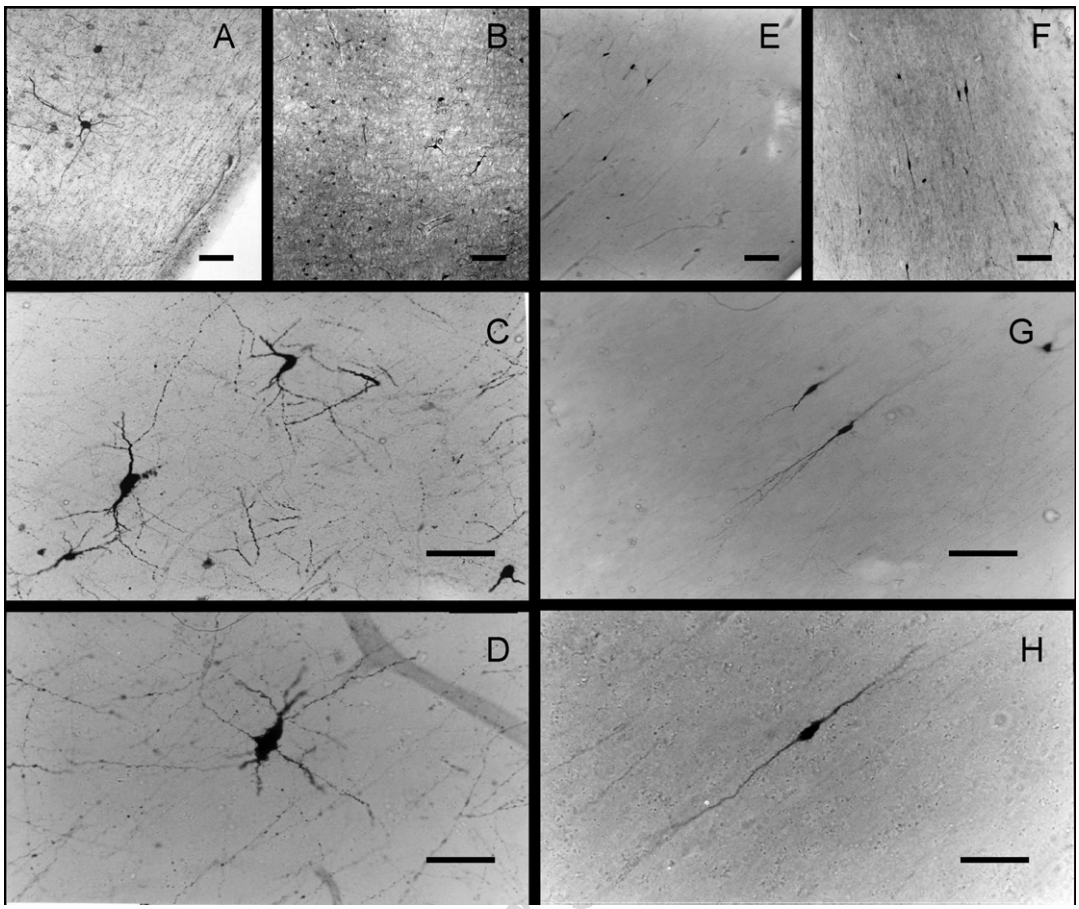

Fig. 2 Vibratome sections of temporal cortex stained with the NADPH diaphorase histochemical technique: (A—D) sections of epileptic patients; (E—H) autopsy control sections. (A and B) low magnification of Ll/ll and LIV/V layers, respectively, showing reactive neurons, (C) intermediate and (D) high magnification of NADPH-d reactive fusiform neurons, located in LVI, showing higher dendritic arborizations. Long beaded fibers may be observed in the field in (C and D). (E and F) low magnification of Ll/ll and LIV/V, respectively, (G) intermediate and (H) high magnification of fusiform NADPH-d reactive neurons located in LVI. Slender dendrites emerge from both neuronal poles. (A, B, E, F, C and G) scale bar: 60 μm. (D and H) scale bar: 30 μm.

Other models of CNS injury as perinatal asphyxia also reported an increase of the cell area of NADPH-d reactive neurons. This enlargement of reactive area could be related to an increase of NO synthesis by the stained ''hypertrophic/cytomegalic'' neurons as adaptative response (Loidl et al., 1997).

The functional and pathological meaning of NO in epilepsy remains controversial. The hyperactivity of NO neurons could contribute to abnormal cortical excitability as NO enhance neurotransmitter release, however, NO could also be involved in adaptative neuroprotective functions as NO can prevent an overstimulation of NMDA receptors (Kato et al., 2005; Ferraro et al., 1999; Noh et al., 2006). Further research is needed to clarify the role of NO during epileptogenesis and to determine the presence of the different NOS isoforms using immunocytochemistry and specific antibodies.

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References

Chung, K.K., Dawson, T.M., Dawson, V.L., 2005. Nitric oxide S-nitrosylation and neurodegeneration. Cell Mol. Biol. 51 (3), 247—254.

Dawson, T.M., Dawson, V.L., 1994. Nitric oxide: actions and pathological roles. The Neurosci. Prev. Issue, 9—21.

Ferraro, G., Montalbano, M.E., La Grutta, V., 1999. Nitric oxide and glutamate interaction in the control of cortical and hippocampal excitability. Epilepsia 40 (7), 830—836.

González-Hernández, T., García-Marín, V., Pérez-Delgado, M.M., González-González, M.L., Rancel-Torres, N., González-Feria, L., 2000. Nitric oxide synthase expression in the cerebral cortex of patients with epilepsy. Epilepsia 41 (10), 1259—1268.

Itoh, K., Watanabe, M., Yoshikawa, K., Kanaho, Y., Berliner, L.J., Fujii, H., 2004. Magnetic resonance and biochemical studies during pentylenetetrazole-kindling development: the relationship between nitric oxide, neuronal nitric oxide synthase and seizures. Neuroscience 129, 757—766.

Kato, N., Sato, S., Yokoyama, H., Kayama, T., Yoshimura, T., 2005. Sequencial changes of nitric oxide levels in the temporal lobes of kainic acid-treated mice following application of nitric oxide synthase inhibitors and phenobarbital. Epilepsy Res. 65 (1/2), 81—91.

Kirkby, D.R., Forbes, R.A., Subramanian, S., 1996. Modification of kainate-induced behavioral and electrographic seizures following inhibition of nitric oxide synthase in mice. Epilepsy Res. 24 (2), 79—90.

Leite, J.P., Chimelli, L., Terra-Bustamante, V.C., Costa, E.T., Assirati, J.A., de Nucci, G., Martins, A.R., 2002. Loss and sprouting of nitric oxide synthase neurons in the human epileptic hippocampus. Epilepsia 43 (Suppl. 5), 235—242.

Loidl, C.F., Capani, F., López-Costa, J.J., Selvin-Testa, A., López, E.M., Pecci Saavedra, J., 1997. Long-term morphological changes in NADPH-diaphorase reactivity in striatal and cortical neurons following experimental perinatal asphyxia: neuroprotective effects of hypothermia. Int. J. Neurosci. 89, 1—14.

Murashima, Y.L., Yoshii, M., Suzuki, J., 2000. Role of nitric oxide in the epileptogenesis of EL mice. Epilepsia 41 (Suppl. 6), S195—S199.

Noh, H.S., Kim, D.W., Cho, G.J., Choi, W.S., Kang, S.S., 2006. Increased nitric oxide caused by the ketogenic diet reduces the onset time of kainic acid-induced seizures in ICR mice. Brain Res. 1075 (1), 193—200.

Osonoe, K., Mori, N., Suzuki, K., Osonoe, M., 1994. Antiepileptic effects of inhibitors of nitric oxide synthase examined in pentylenetetrazol induced seizures in rats. Brain Res. 663, 338—340.

Vincent, S.R., Kimura, H., 1992. Histochemical mapping of nitric oxide synthase in the rat brain. Neuroscience 46 (4), 775—784.

Yasuda, H., Masami, F., Fujisawa, H., Ito, H., Suzuki, M., 2001. Changes in nitric oxide synthesis and epileptic activity in the contralateral hippocampus of rats following intrahippocampal kainate injection. Epilepsia 42 (1), 13—20.