Effect of Oxidative Stress on (^{3}H)N-Methylscopolamine Binding and Production of Thiobarbituric Acid Reactive Substances in Rat Cerebral Cortex Membranes.

Z. KVALTÍNOVÁ, I. JURÁNEK, J. MACHOVÁ and S. ŠTOLC

Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dúbravská cesta 9, 842 16 Bratislava, Slovakia

Abstract. We investigated the effect of lipid peroxidation, *in vitro* induced by H_2O_2 or $FeSO_4$ and ascorbic acid, on binding properties of muscarinic receptors in rat cerebral cortex membranes. Simultaneously the concentrations of thiobarbituric acid reactive substances (TBARS) were measured to assess the extent of lipid peroxidation. In conditions of increased TBARS levels the density of (^{3}H)N-methylscopolamine [(^{3}H)NMS] binding sites in rat cerebral cortex membranes was not affected. Decreased numbers of (^{3}H)NMS binding sites observed in the presence of high concentrations of H_2O_2 (100 and 1000 mmol.l^{-1}) accompanied by a decrease of TBARS levels might be related to a nonspecific effect of H_2O_2 on cellular proteins.

Key words: Muscarinic binding sites — Oxidative stress — Cerebral cortex membranes

Introduction

Several reports have appeared during recent years, concerning binding properties of membrane receptors for catecholamines (Kramer et al. 1987; Heikilla 1984), serotonin (Muakkassak-Kelly et al. 1982, Villacara et al. 1989) and GABA (Yoneda et al. 1985) in brain tissue under conditions in which increased amounts of free oxygen radicals are generated. It appears that altered cholinergic transmission is involved in some mental changes that occur with aging and in different neurological disorders (Mesulam et al. 1983) in which an increase of free oxygen radical generation has been suggested (Jesberger and Richardson 1991). The aim of this study was to investigate the effects of lipid peroxidation on muscarinic binding sites in the rat cerebral cortex membranes.

Materials and Methods

Preparation of tissue for binding studies

Male Wistar rats (300–350 g) were decapitated and whole brains without cerebellum were rapidly removed and placed into ice cold NaCl solution (153.87 mmol.l^{-1}). The cerebral cortex was dissected, weighed and immediately frozen in liquid nitrogen. The tissue was thawed on the day of the binding assay. The samples were homogenized (Ultra-Turrax) in ice cold 5 mmol.l^{-1} Tris-HCl buffer with 1 mmol.l^{-1} $MgCl_2$ at pH 7.4 (100 mg tissue/1 ml buffer). The homogenates were centrifuged at $1000 \times g$ for 10 min at 4 °C. The supernatant was centrifuged three times at $50,000 \times g$ for 10 min with intermittent resuspension of the pellet. The final pellet was resuspended in 10 volumes of ice cold 50 mmol.l^{-1} Tris-HCl buffer.

Lipid peroxidation in vitro

Rat cerebral cortex membranes prepared as mentioned above were preincubated with the oxygen radical generating system: (1) 60 min at 37 °C with H_2O_2 in concentrations ranging from 10 to 1000 mmol.l^{-1} (Olafsdottir et al. 1991; Yoneda et al. 1985) or (2) 30 min at 37 °C with ferrous sulfate (70 μmol.l^{-1}) and ascorbic acid (5 μmol.l^{-1}) (Wills 1969; Horáková et al. 1990). The ferrous sulfate and ascorbic acid solutions were freshly prepared and mixed immediately before being added to each sample.

Control membrane samples corresponding to the first and second group were preincubated with 50 mmol.l^{-1} Tris-HCl buffer for 60 and 30 min respectively.

Binding assays for (^{3}H)N-methylscopolamine [(^{3}H)NMS]

In saturation experiments, preincubation with an oxygen radical generating system as above, was followed by incubation of samples for 30 min at 37 °C with increasing concentrations of (^{3}H)NMS (74 Ci.mmol.l^{-1}; 0.1 – 8 nmol.l^{-1}). Nonspecific binding was assessed in the presence of 10 μmol.l^{-1} atropine. The final volume of a sample was 250 μl. After the incubation, 5 ml of ice cold 50 mmol.l^{-1} Tris-HCl buffer was added to each sample and the sample was immediately filtered under vacuum (Millipore) through glass fiber filters (Whatman GF/C). The filters were additionally washed two times with 5 ml of the buffer, transferred to tubes and dried in a oven at 60 °C for 60 min. Then, 10 ml of SLT scintillation fluid was added to each tube with the sample on the filter and radioactivity of the samples was counted after 20 h (Packard TriCarb). The proteins, about 0.1–0.2 mg per sample, were determined by the method of Lowry et al. (1951) with bovine albumin as standard.

Assay of thiobarbituric acid reactive substances (TBARS)

The extent of lipid peroxidation in rat cortical membranes after their preincubation with H_2O_2 or $FeSO_4$ and ascorbic acid was assessed by measuring the level of TBARS (Scherer and Deamer 1986).

Data analysis

The saturation curves of specific binding of (^{3}H)NMS to muscarinic binding sites were analyzed by fitting experimental data to nonlinear function, and Scatchard plots were analyzed by fitting experimental data to linear function, with both procedures based on the method of least squares (Munson 1984). The K_D and B_{max} values, which are expressed as mean values with 95% intervals of confidence, were obtained by Scatchard analysis.

Chemicals

Chemicals used were as follows: atropine sulfate (Spofa), ferrous sulfate (Sigma), malondialdehyde bis-acetal (Merck), 1-(N-methyl-^{3}H) scopolamine methyl chloride [(^{3}H)NMS] (Amersham), scintillation fluid SLT 41 (Chemapetrol), thiobarbituric acid (Sigma), trichloroacetic acid (Merck), tris(hydroxymethyl)aminomethane [Tris] (Serva). All other chemicals were obtained from Lachema.

Results

Effect of hydrogen peroxide on (^{3}H)NMS binding and TBARS level

In the control group, rat cerebral cortex membranes were preincubated for 60 min with Tris-HCl buffer and then incubated for 30 min with (^{3}H)NMS (0.1–8 nmol.l^{-1} in the presence or absence of 10 μmol.l^{-1} atropine. The specific binding of (^{3}H)NMS to muscarinic binding sites was saturable with a B_{max} value of 5.66 (4.66–6.66) pmol.mg^{-1} protein (Fig. 1) and with a K_D value of 0.54 (0.15–0.92) nmol.l^{-1} ($n = 6$). Nonspecific binding did not exceed 5% of the total binding. The changes in specific binding of (^{3}H)NMS to muscarinic binding sites of rat cerebral cortex membranes in samples preincubated for 60 min with increasing concentrations of H_2O_2 (10; 100; 1000 mmol.l^{-1}) are presented in Fig. 1. Preincubation of membranes with 10 mmol.l^{-1} of H_2O_2 did not elicit any change in the density of (^{3}H)NMS binding sites. In the presence of similar concentrations of H_2O_2 (6 to 12 mmol.l^{-1}) a significant increase of TBARS level was found (Fig. 1).

Preincubation of rat cerebral cortex membranes with 100 or 1000 mmol.l^{-1} of H_2O_2 reduced significantly the density of (^{3}H)NMS binding sites (by about 43 and 95% respectively). In the presence of a similar range of concentrations of H_2O_2 (100–300 mmol.l^{-1}) the level of TBARS was significantly lower than in the control group (Fig. 1). After preincubation of membranes with 1000 mmol.l^{-1} of H_2O_2, TBARS were not detectable.

K_D values were not significantly changed after preincubation of membranes with any concentration of H_2O_2 used (10; 100; 1000 mmol.l^{-1}) (not shown).

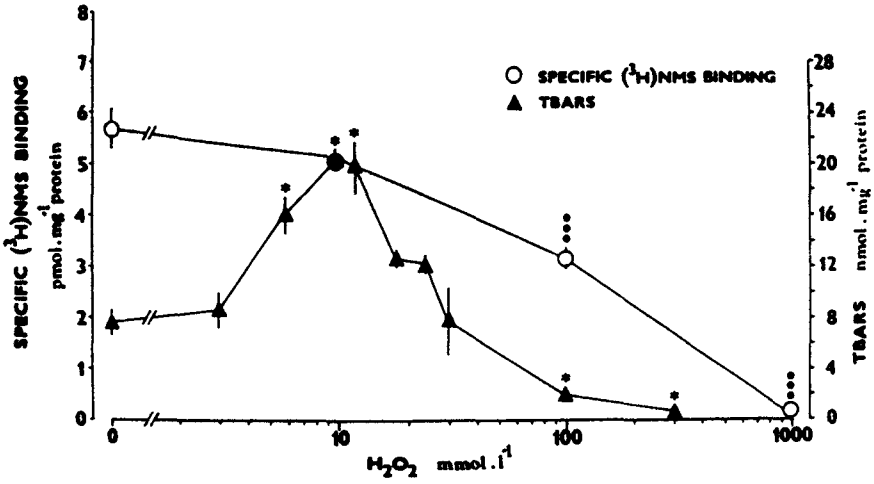

Figure 1. Effects of hydrogen peroxide (3–1000 mmol.l^{-1}) on TBARS level (▲) and on B_{max} of (^{3}H)NMS (0.1–8 nmol.l^{-1}) specific binding (○) to muscarinic binding sites of rat cerebral cortex membranes. Membranes were preincubated with various concentrations of H_2O_2 at 37 °C for 60 min and then (^{3}H)NMS binding and the level of TBARS were measured. Each value represents the mean ± S.E.M. obtained from 3 to 6 experiments. $^*p < 0.05$; $^{***}p < 0.001$, compared to control values.

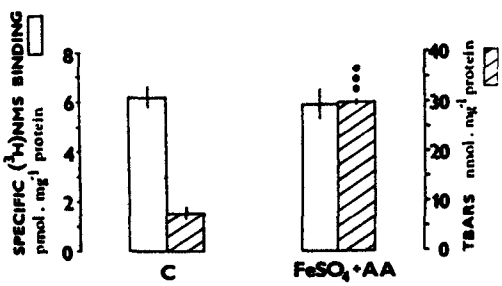

Figure 2. Effects of ferrous sulfate ($FeSO_4$; 70 μmol.l^{-1}) and ascorbic acid (AA; 5 μmol.l^{-1}) on the level of TBARS (▨) and the B_{max} of (^{3}H)NMS (0.1–8 nmol.l^{-1}) specific binding (□) to muscarinic binding sites of rat cerebral cortex membranes. Membranes were preincubated with $FeSO_4$ and ascorbic acid at 37 °C for 30 min and then (^{3}H)NMS binding and TBARS level were measured. Each value represents the mean ± S.E.M. obtained from 3 to 5 experiments. $^{***}p < 0.001$, compared to control values.

Effect of ferrous sulfate and ascorbic acid on (^{3}H)NMS binding and TBARS level

Rat cerebral cortex membranes were preincubated with Tris-HCl buffer for 30 min and then incubated for 30 min with (^{3}H)NMS (0.1–8 nmol.l^{-1}) in the presence or absence of 10 μmol.l^{-1} atropine. The B_{max} value for (^{3}H)NMS specific binding was 6.21 (4.94–7.49) pmol.mg^{-1} protein and the K_D value was 0.66 (0.29–1.02) nmol.l^{-1} ($n = 5$). After 30 min preincubation of membranes with 70 μmol.l^{-1} $FeSO_4$ and 5 μmol.l^{-1} ascorbic acid no significant changes were found in the density (Fig. 2) or affinity (not shown) of (^{3}H)NMS binding sites, compared to the control values.

Preincubation of rat cortical membranes with 70 μmol.l^{-1} $FeSO_4$ and 5 μmol.l^{-1} ascorbic acid for 30 min resulted in an approximately fourfold increase in the concentration of TBARS (Fig. 2).

Discussion

In the brain tissue a decrease in the density of adrenergic (Heikilla 1984; Kramer et al. 1987) and serotoninergic receptors (Villacara et al. 1989; Muakkassak-Kelly et al. 1982) was observed in conditions supposed to be associated with increased amounts of free oxygen radicals. In our experiments, there were no significant changes in the density of muscarinic binding sites of rat cerebral cortex membranes observed in the presence of H_2O_2 in a concentration inducing the largest increase in TBARS indicating lipid peroxidation. Neither did the presence of the other system studied, $FeSO_4$ and ascorbic acid, induce changes in binding properties of muscarinic receptors of the rat cerebral cortex membranes, although an increase in TBARS concentration occurred. Our results are in agreement with those of Gajewski and coworkers (1988) who did not observe significant changes in the density of muscarinic binding sites on different types of tissues, in rat myocytes and lymphocytes, pretreated with H_2O_2. Similarly, Van der Vliet and coworkers (1989) concluded on the basis of their experiments on the isolated intestine that muscarinic receptors were not specifically damaged by 1 mmol.l^{-1} H_2O_2 or 0.5 mmol.l^{-1} cumene hydroperoxide. On the other hand, Arora and Hess (1985) showed the cardiac muscle sarcolemma to exhibit a significant decrease in the level of muscarinic receptors after H_2O_2 (4.41 - 441 mmol.l^{-1}) treatment. In our experiments H_2O_2 in concentrations of 100 and 1000 mmol.l^{-1} caused a marked decrease in the density of (^{3}H)NMS binding sites. These concentrations of H_2O_2, however, appear to be very high as they exceed those observed in the vicinity of activated leukocytes (Fligiel et al. 1984). The decreased densities of muscarinic binding sites induced by H_2O_2 at 100 and 1000 mmol.l^{-1} were accompanied by decreased concentrations of TBARS which could be due to degradation of malondialdehyde in the presence of high H_2O_2 concentrations (Kostka and Kwan 1989). The deleterious effect of high H_2O_2 concentrations on muscarinic receptor binding capacity

could be due to a nonspecific action on cellular proteins.

Differences in the experimental conditions employed for the generation of oxygen free radicals, different types of radioligands used and species and tissue dependence might have contributed to the divergence of reported effects on receptor density (Kaneko et al. 1991).

In conclusion, the possibility should be considered that in comparison with other types of neurotransmitter receptors, muscarinic receptors in the brain may be less sensitive to conditions supposed to be associated with increased amounts of free oxygen radicals and lipid peroxidation.

References

Arora R. C., Hess M. L. (1985): Effect of reduced oxygen intermediates on sarcolemmal muscarinic receptors from canine heart. Biochem. Biophys. Res. Commun. **130,** 133—140

Fligiel S. E. G., Lee E. C., McCoy J. P., Johnson K. J., Varani J. (1984): Protein degradation following treatment with hydrogen peroxide. Amer. J. Pathol. **115,** 418—425

Gajewski M., Laskowska-Bozek H., Orlewski P., Maslinski S., Ryzenski J. (1988): Influence of lipid peroxidation and hydrogen peroxide on muscarinic cholinergic receptors and ATP level in rat myocytes and lymphocytes. Int. J. Tissue React. **10,** 281—289

Heikilla R. E. (1984): Oxygen Radicals in Chemistry and Biology. Walter de Gruyter & Co., Berlin-New York

Horáková Ľ., Juránek I., Bokníková B. (1990) : Antioxidative effect of stobadine on lipid peroxidation of rat brain mitochondria. Biológia (Bratislava) **45,** 313—318

Jesberger J. A., Richardson J. S. (1991) : Oxygen free radicals and brain dysfunction. Int. J. Neurosci. **57,** 1—17

Kaneko M., Chapman D. C., Ganguly P. K., Baemish R. E., Dhalla N. S. (1991): Modification of cardiac adrenergic receptors by oxygen free radicals. Amer. J. Physiol. **260,** H821—H826

Kostka P., Kwan Ch.-Y. (1989): Instability of malondialdehyde in the presence of H_2O_2: Implications for the thiobarbituric acid test. Lipids **24,** 545—549

Kramer K., Voss H.-P., Grimbergen J. A., Timmerman H., Bast A. (1987): The effect of ischemia and recirculation, hypoxia and recovery on anti-oxidant factors and beta-adrenoceptor density. Biochem. Biophys. Res. Commun. **149,** 568—575

Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. (1951): Protein measurements with the Folin phenol reagents. J. Biol. Chem. **193,** 265—275

Mesulam M.-M., Mufson E. J., Wainer B. H., Levey A. I. (1983): Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch1-Ch6). Neuroscience **10,** 1185—1201

Munson P. J. (1984): Heterogenous receptors and binding curve analysis in neurobiology. In: Brain Receptor Methodologies. (Eds. P.J. Marangos et al.) pp. 33—49, Academy Press, Orlando

Muakkassak-Kelly S. F., Andersen J. W., Shis J. C., Hochstein P. (1982): Decreased (^{3}H)serotonin and (^{3}H)spiperone binding consequent to lipid peroxidation in rat cortical membranes. Biochem. Biophys. Res. Commun. **104,** 1003—1010

Olafsdottir K., Ryrfeldt A., Atzor L., Berggreen M., Moldeus P. (1991): Hydroperoxide-induced broncho- and vasoconstriction in the isolated rat lung. Exp. Lung Res. **17,** 615—627

Scherer N. M., Deamer D. W. (1986): Oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulfhydryl groups in the Ca^{2+} ATP-ase. Arch. Biochem. Biophys. **246,** 589—601

Van der Vliet A., Tuinstra T. J. R., Bast A. (1989): Modulation of oxidative stress in the gastrointestinal tract and effect on rat intestinal motility. Biochem. Pharmacol. **38,** 2807—2818

Villacara A., Kuman K., Yamamoto T., Mrsulja B. B., Spatz M. (1989): Ischemic modification of cerebrocortical membrane 5-hydroxytryptamine receptors, fluidity and inducible *in vitro* lipid peroxidation. J. Neurochem. **53,** 595—601

Wills E. D. (1969): Lipid peroxide formation in microsomes. General considerations. Biochem. J. **113,** 315—324

Yoneda Y., Kuriyama K., Takahashi M. (1985): Modulation of synaptic GABA receptor binding by membrane phospholipids: possible role of active oxygen radicals. Brain Res. **333,** 111—122

Final version accepted January 28, 1993