PHYSIOLOGY

Negative Regulation of Caspase-3 Expression in the Neonatal Cerebral Cortex by α_{2A}-Adrenoceptors

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Antisense oligonucleotide to α_{2A}-adrenoceptors increased the levels of mRNA (reverse polymerase chain reaction) and protein for a key executioner protease of apoptosis caspase-3 (immunoblotting assay) in the cerebral cortex of newborn rats. The relationship between the observed effect and low expression of α_2-adrenoceptors was confirmed by the possibility of correcting this phenomenon by clonidine (stimulator of α_2-adrenoceptors).

Key words: *α_2-adrenoceptors; caspase-3; brain; ontogeny*

More than 50% neurons undergo apoptosis in developing mammalian brain [8]. The intensity of physiological apoptosis is modulated by various neurotransmitters, including norepinephrine (via specific α_2-adrenergic receptors, α_2-AR) [4]. However, the data on the influence of these AR on viability of brain cells are contradictory. Some authors reported that α_2-AR stimulated cell death [5,7], while others showed that α_2-AR agonists prevented this process [6,13]. This discrepancy is probably related to differences in the effect of the receptor on cell viability in various brain regions. Clonidine (α_2-AR-stimulating agent) increases the level of mRNA for caspase-3 (major protease of apoptosis in nervous cells) in the brainstem, but not in the cortex of neonatal rats [4]. Hence, α_2-AR do not regulate caspase-3 expression in the cortex, otherwise the receptor-mediated negative or positive regulation of protease expression in this brain region is effected at the maximum level due to interaction of existing receptor molecules with available endogenous norepinephrine.

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In our study, the effect of long-term modulation of receptor expression was evaluated by sequence-specific inhibition of target gene expression at the level of caspase-3 expression in the brain cortex of neonatal rats.

MATERIALS AND METHODS

Experiments were performed on Wistar rat pups. Antisense deoxyoligonucleotide phosphorothioate (antisense) to α_2-AR (agcccatgggcgcaaagc, 0.36 μg/5 μl per day) was administered into the brain of cold-anesthetized rats on days 2, 3, and 4 of life (1 mm caudal to lambda, 5.5 mm below the skull surface). Control animals received random sequence oligonucleotide (gacgacccagtgagcacg) or physiological saline via the same route. Clonidine (Serva) in a dose of 0.1 or 4.0 μg/kg was injected subcutaneously on day 5 of life. The cortex (anterior half of the hemisphere surface, 1.5 mm thick) was isolated on day 6 of life. The levels of caspase-3 mRNA and protein were measured in tissue samples.

mRNA content in total RNA was estimated by means of a semiquantitative reverse polymerase chain reaction (PCR). Total RNA was isolated by the single-step guanidine isothiocyanate method. cDNA

was isolated with oligo-dT primer and MuLV revertase (SibEnzim). cDNA regions specific for each gene were amplified by the standard method [2] using primers for caspase-3 [12] or β-actin [9]. PCR products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide. The number of PCR cycles for each pair of primers corresponded to the exponential phase of accumulation of optically detected amplification product. For all genes the amount of PCR product linearly depended on amounts of cDNA.

The study of caspase-3 protein involved homogenization of tissue samples in lysing buffer with 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin. After centrifugation at 14,000*g* and 4°C for 15 min, 50 μg supernatant protein were denatured in a buffer (50 mM Tris-HCl, pH 6.8; 10% glycerol; 100 mM 2-β-mercaptoethanol; 1% sodium dodecyl sulfate; and 0.002% bromophenol blue) at 95°C for 5 min, separated by electrophoresis (VE-2 system, Helicon) in 15% polyacrylamide gel with sodium dodecyl sulfate, and transferred (Trans-Blot system, Bio-Rad Laboratories) to a nitrocellulose membrane (Nitro). Caspase-3- and actin were visualized using primary (H-277;1:250 and I-19-R; 1:1000, respectively) and secondary rabbit polyclonal antibodies conjugated with alkaline phosphatase. They were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitrotetrazolium blue. The signals of caspase-3 and actin linearly depended on total protein content.

The levels of caspase mRNA and protein were estimated relative to the levels of actin mRNA and protein in the sample. The study was performed after scanning of gels and membranes (BioDoc II, Biometra GmbH) by means of computerized densitometry (Scion Image).

The effects of treatment on study parameters were evaluated by two-factor analysis of variance (ANOVA). The significance of intergroup differences was estimated by a multiple comparison test (LSD).

RESULTS

Antisense to α_{2A}-AR significantly inhibited the expression of the target gene, which was manifested in a decrease in the level of receptor mRNA and number of receptor protein molecules (test for specific binding of ^{3}H-RX821002) [10,11]. Antisense decreased the level of receptor mRNA and receptor density in the frontal cortex of rat pups by more than 2 times and one-third, respectively, compared to animals receiving control oligonucleotide or physiological saline) [10,11].

The levels of mRNA and protein for the key executioner protease of apoptosis caspase-3 increased in the cortex of newborn rats after intracerebral administration of antisense to α_{2A}-AR (Fig. 1). Caspase-3 mRNA is more sensitive to blockade of AR expression with antisense compared to enzyme protein. mRNA level increased nearly by 2 times in animals receiving antisense and not subjected to clonidine treatment, while protein content increased only by one-third. α_2-AR-Stimulator clonidine in

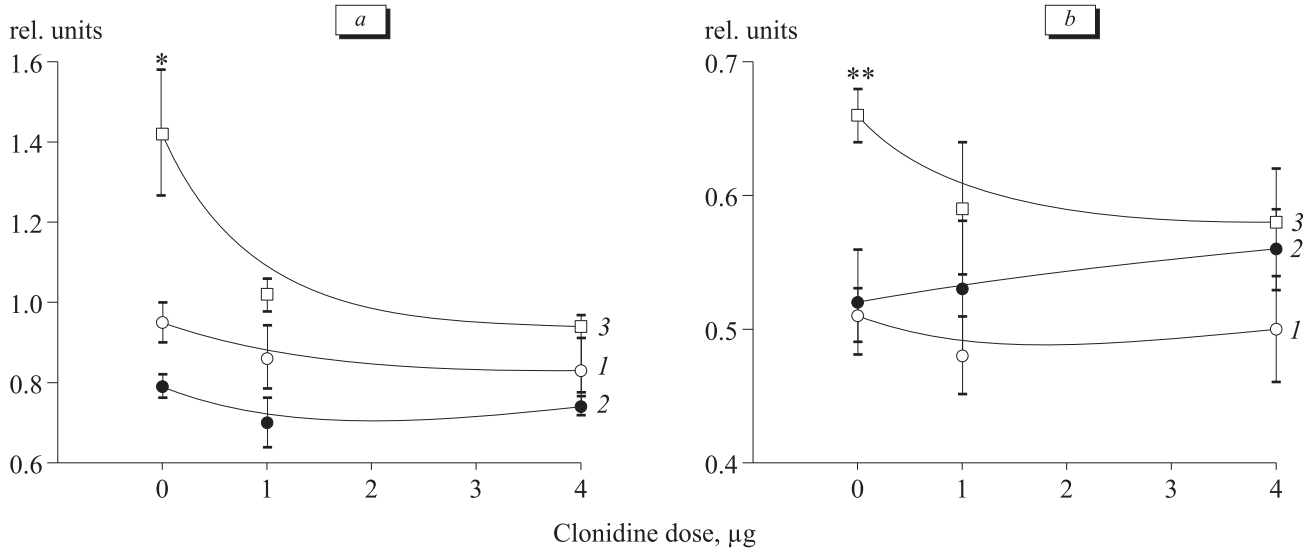

Fig. 1. Levels of caspase mRNA (*a*) and protein (*b*) relative to the levels of β-actin mRNA and protein in the cerebral cortex of 6-day-old rat pups after intracerebral administration of antisense to α_{2A}-AR (days 2-4 of life) and compensatory pharmacological stimulation of these AR by subcutaneous injection of clonidine (day 5 of life). *F(2.69)=6.84; $p<0.002$; **F(2.37)=6.42; $p<0.005$. *1*) solvent, *2*) control, *3*) antisense.

both doses had no effect on the levels of caspase-3 mRNA and protein in rat pups receiving control oligonucleotide or physiological saline on days 2-4 of life. However, replacement therapy for low expression of α_{2A}-AR with receptor-stimulating agent clonidine decreased the levels of caspase-3 mRNA and protein that were elevated after antisense treatment. Similarly to antisense, clonidine produced a more potent compensatory effect on caspase-3 mRNA (this parameter decreased by 1.5 times) and practically abolished the effect of antisense. After treatment with clonidine, protease protein content in animals receiving antisense did not differ from that in controls.

The observed effects are related to inhibition of the expression of the target gene. Our conclusion is derived from the data that control oligonucleotide does not cause these changes. Moreover, administration of receptor-stimulating agent clonidine compensated for low expression of α_2-AR. The effects of oligonucleotide were verified pharmacologically with such targets for sequence-specific inhibition of expression as receptor genes. Their proteins usually have specific low-molecular-weight stimulating agents (*e.g.*, clonidine for α_2-AR). This verification does not appear an unreasonable procedure, because oligonucleotide-induced changes can include not only inhibition of target gene expression [1].

Our experiments demonstrate negative regulation of caspase-3 expression in the neonatal cerebral cortex with α_{2A}-AR. This "hidden" function becomes most pronounced during the interaction of the receptor with endogenous norepinephrine. Published data show that stimulation of receptors with clonidine is followed by protease induction in the brainstem region [4]. However, this exogenous ligand in the doses used in our study or previous experiments [4] has no effect on caspase-3 expression. Regional differences in the function of α_2-AR are probably related to the ability of these receptors to couple with various intracellular signal transduction systems [3]. The distribution of these systems differs in the cerebral cortex and brainstem. Moreover, they can induce or inhibit protease expression in the developing brain.

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