A Novel Brain-Targeted Antioxidant (AD4) Attenuates Haloperidol-Induced Abnormal Movement in Rats:

Implications for Tardive Dyskinesia

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Background: Tardive dyskinesia (TD), characterized by abnormal movements, is the major late-onset chronic side effect of antipsychotic treatment found in about 30% of those patients. The association of oxidative stress and the release of free radicals is one of the hallmarks of dopaminergic malfunctions and is one of the leading theories suggested for the pathophysiology of TD. To this day, no brain-targeted antioxidant has been tested as a potential treatment of TD. In light of this assumption, the authors chose a novel, low-molecular weight thiol antioxidant, N-acetyl cysteine amide (AD4), that crosses the blood–brain barrier as a possible treatment of TD.

Objective: To examine the protective effects of the novel brain-penetrating antioxidant AD4 on TD experimental models.

Methods: The typical vacuous chewing movement occurs in rats following chronic haloperidol injections (1.5 mg/kg/day intraperitoneally for 21 days). This purposeless mouth opening in the vertical plane is similar to TD symptoms in humans. The authors tested rats treated with haloperidol without or with AD4 in the drinking water (1 g/kg orally). Thiobarbituric acid reactive substances and anti-carbonyl antibodies were used to measure oxidation of membranes and proteins.

Results: Haloperidol increased the vacuous chewing movements to 66.5 ± 7.6 movements/5 minutes compared with 16.4 ± 2.4 movements/5 minutes in untreated rats ($P < 0.01$). Coadministration of haloperidol and AD4 decreased the vacuous chewing movements level to 42.1 ± 6.7 movements/5 minutes ($P < 0.05$). Haloperidol also increased the level of lipid peroxidation and protein oxidation in the rat brain, whereas coadministration with AD4 preserved their normal levels.

Conclusion: Haloperidol causes behavioral abnormalities associated with oxidative stress in rats, similar to TD. AD4, the brain-targeted potent antioxidant, reduces the cellular oxidation markers and improves the typical clinical behavior. Hence, AD4 is a potential new treatment of antipsychotic-induced TD.

Key Words: oxidative stress, antioxidants, AD4, tardive dyskinesia, vacuous chewing movement

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Tardive dyskinesia (TD) is a common, disabling, socially embarrassing, and potentially irreversible complication of chronic treatment with antipsychotics.[1] The precise cause for the emergence of the involuntary movements is unknown and may be linked to long-term inhibition of striatal dopamine receptors by these agents, leading to receptorial supersensitivity and other postsynaptic perturbations.[2,3] It has also been postulated that oxidative stress induced by the antipsychotic agents has an important pathogenetic role.[4] These compounds are toxic to neuronal cultures through excess formation of free radical species.[5] Damage can be prevented by cotreatment with antioxidants.[6,7] Chronic systemic administration of haloperidol in rats enhances lipid peroxidation and reduces the natural intracellular antioxidant mechanisms such as glutathione (GSH), superoxide dismutase, and catalase in brain and liver.[8,9] Consequently, treatment with antioxidants such as vitamin E, melatonin, and bioflavonoids were tried in TD with varying degrees of success.[10,11] However, most currently available antioxidants do not cross the blood–brain barrier (BBB) after systemic administration or do so inefficiently.[12]

We have recently prepared the amide form of N-acetylcysteine, N-acetylcysteine amid (AD4), a novel, thiol-containing, low-molecular weight brain-targeted antioxidant that scavenges reactive oxygen species, chelates copper ions, and restores intracellular GSH.[13] We have already shown that treatment with AD4 was effective in preventing and suppressing experimental autoimmune encephalomyelitis, a model of human multiple sclerosis.[14] We also found that it prevents dopaminergic neuronal damage in several animal models of Parkinson disease.[15] To summarize, we have shown in our previous research that AD4 is a low-molecular weight molecule and potent antioxidant. Its uniqueness is due to the neuroprotective qualities and the ability to penetrate the BBB, and it serves as a centrally acting antioxidant.[13–17] We now report that treatment with AD4 prevents haloperidol-induced vacuous

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chewing movements (VCMs) in an experimental model of TD in rats.[18]

MATERIALS AND METHODS

Animals

Thirty female Wistar rats weighing 200 to 250 g (10 in each group), obtained from Tel-Aviv University, Israel, were housed under standard laboratory conditions—constant temperature (22 ± 1°C), humidity (relative, 25%) and a 12-hour light/dark cycle—and were allowed free access to food and water. The animals and protocol procedures were approved and supervised by the Animal Care Committee of Tel-Aviv University.

Treatment

Haloperidol (RBI) was dissolved in lactic acid 0.1% (Sigma) and administered (1.5 mg/kg, intraperitoneally [IP]) chronically for 21 days.[9] AD4 was synthesized as described by Atlas et al,[17] dissolved in drinking water, and administered chronically for 21 days (1 g/kg/day orally). It should be mentioned here that AD4 slowly hydrolyzes in the water and therefore high concentrations of AD4 were used.

Behavioral Testing

Open Field Test

Rats were placed in a box (20 × 60 × 30 cm) divided into 6 squares by lines on the floor, and the line crossings were observed and counted during a period of 5 minutes.

Orofacial Dyskinesia

Assessment of orofacial dyskinesia was done on day 22, 24 hours after the last haloperidol and/or AD4 treatment. Rats were placed individually in a transparent cage in which mirrors were positioned below and behind to enable observation of oral dyskinesia when the rat was not facing the observer. The rats were allowed 5 minutes of habituation to the observation cage before behavioral assessments. To quantify the occurrence of oral dyskinesia, hand-operated counters were used to record VCM. VCMs were defined as single mouth openings in the vertical plane not directed toward physical material. If VCMs occurred during a period of grooming, they were not taken into account. The behavioral parameters of oral dyskinesia were measured continuously for a period of 5 minutes. In all the experiments the scorer was blinded and unaware of which treatment was given to the animals.

Biochemical Analysis

Lipid Peroxidation

The thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde (MDA) equivalents, were used as a measure for lipid peroxidation.[19] In brief, posterior portions that included the right striata, midbrain, and the right cerebellar hemisphere were homogenized in PBS (1:7) on ice. After centrifugation, an aliquot of the suspension was taken for a protein assay using the BCA protein assay (Pierce, IL). A total of 250 μL of each specimen was mixed with 3% SDS (Sigma), and 250 μL TBA reagent (50 μL trichloroacetic acid [Sigma], 1.675 mg thiobarbituric acid [Sigma]) was added to each tube and "vortexed." The reaction mixture was incubated at 90°C for 20 minutes and stopped on ice. After cooling to room temperature, TBARS were extracted with 0.5 mL n-butanol and separated by centrifugation at 3,000 *g* for 5 minutes. Total TBARS was measured by absorbance at 532 nm in an ELISA reader (SLT Labinstrument 400ATC). MDA (Aldrich) dilutions were used as standards. The standard measurements were performed in duplicate and the results were expressed as micromolecular equivalents of MDA per microgram protein.

Protein Oxidation

Carbonyls levels were measured with the OxyBlot kit (Intergen, Finland). Briefly, posterior portions that included the left striata, midbrain, and the left cerebellar hemisphere from 5 rats from each group were homogenized in a protease inhibitor buffer. After quantifying protein amounts in suspension using the BCA protein assay, we pooled 10 μg protein from each sample to generate 3 samples (one for each tested group) containing 10 μg protein each. According to the manufacturer's protocol, these samples were mixed with an equal volume of 12% SDS and 2 volumes of 2,4-dinitrophenylhydrazine (DNPH × 1) solution. Control reactions used derived control solution instead of the DNPH solution. The reaction was allowed to proceed for 15 minutes at room temperature and was stopped by the addition of 1.5 volumes of neutralizing solution, and then separated on 3 to 10% polyacrylamide gels and transferred to PVDF membrane (Amersham, UK). After blocking, using PBS solution with Tween-20 (0.01% [PBS-T], Sigma) and BSA (1%, Sigma), the membrane was exposed to primary antibody (rabbit anti-DNP), specific to the DNP moiety of the proteins overnight at 4°C. The membrane was washed with PBS-T and incubated (1 hour at room temperature) with the secondary antibody (goat antirabbit IgG, 1:300). After a 15-minute and 2 10-minute washes in PBS-T, the proteins were detected using the Supersignal west pico Chemiluminescent (Pierce, IL), developed and analyzed using VersaDoc and QuantityOne software (version 4.3.0, BioRad). Later, the membrane was stripped and exposed to mouse antiactin I antibody (1:1,000, Chemicon) and incubated with goat antimouse antibody (1:20,000, Jackson) to normalize the results.

Statistical Analysis

All data are presented as means ± SEM. The differences between groups were analyzed using one-way analysis of variance (SPSS, version 11.5), with Scheffe post hoc tests for multiple comparisons in each case. In all tests, significance was assigned when $P < 0.05$.

RESULTS

The beneficial effect of AD4 was examined in the VCM model of TD in 3 groups of rats (n = 10, each) as follows: the first group, control, was treated with vehicle (lactic acid 0.1% IP), the second group was treated chronically with haloperidol, (1.5 mg/kg/day IP, once daily for 21 days), and the third group was treated with haloperidol as in the second group, combined with AD4 (1 g/kg/day orally via drinking water). In the first

behavioral test a simplified open field assay was used 30 minutes after haloperidol treatment, on day 19 of the experiment. We found that administration of haloperidol decreased line crossing in the open field test in a significant manner, when compared with the vehicle-treated group (8.89 ± 1.32 vs. 16.1 ± 3.83 respectively, $P < 0.05$). The group of rats given haloperidol combined with AD4 was comparable with the haloperidol alone-treated group (9.2 ± 0.96), indicating that the addition of AD4 did not change the sedative effect of haloperidol.

In the VCM test, the haloperidol-treated group showed a high rate of induced VCM compared with the control group (66.5 ± 7.6 vs. 16.4 ± 2.4 respectively, $P < 0.01$), whereas coadministration of haloperidol and AD4 decreased the induced VCM by 37% to 42.1 ± 6.7 ($P < 0.05$ vs. haloperidol and $P < 0.01$ vs. the control group, Table 1).

Biochemical analysis revealed that in haloperidol-treated rats, levels of brain lipid peroxidation were significantly higher, as measured by the TBARS assay: 0.39 ± 0.07 mM MDA/μg protein versus 0.25 ± 0.1 mM MDA/μg protein in the untreated rats ($P < 0.05$). However, cotreatment with AD4 inhibited lipid peroxidation to 0.26 ± 0.07 ($P < 0.05$ vs. haloperidol), which was comparable with untreated rats (Table 1). Carbonyl levels, used as an indicator for protein oxidation, were detected in the brain extracts by Western blot analysis using anti-DNP antibodies. We found that haloperidol induced an elevation in the carbonyl levels compared with the vehicle-only control group (116.72%), whereas in rats co-treated with haloperidol and AD4, the carbonyl levels were reduced to a third of the vehicle-only group (34.22%, Table 1).

DISCUSSION

Our study shows that chronic treatment with haloperidol increases indices for oxidative stress in rat brain (ie, TBARS [for lipid peroxidation] and carbonyls [for protein oxidation]), confirming previous findings of enhanced levels of reactive oxygen species in the CNS under antipsychotic therapy. The haloperidol-induced increases in brain TBARS and carbonyls were prevented by cotreatment with the BBB-penetrating antioxidant AD4, indicating its effectiveness as a centrally acting free radical scavenger. More important, treatment with AD4 significantly suppressed a haloperidol-induced increase in VCM in rats.

TABLE 1. Behavioral and Biochemical Effect of Haloperidol and AD4

Group	VCM/5 min	Open Field, No. of Lines Crossed	TBARS, mM MDA/μg Protein	Carbonyls, % of Control
Control	16.45 ± 2.49	16.1 ± 3.83	0.25 ± 0.1	100
Haloperidol*	66.55 ± 7.64§	8.89 ± 1.32	0.39 ± 0.07‡*	116.72
Haloperidol* + AD4†	42.1 ± 6.75‡	9.2 ± 0.96	0.26 ± 0.07§	34.22

*Dose of 1.5 mg/kg/day for 21 days; †Dose of 1 g/kg/day orally for 21 days; ‡$P < 0.05$; §$P < 0.01$.

AD4 may act primarily as a radical scavenger. It also hydrolyzes in the CNS to N-acetylcysteine, a potent antioxidant that has a poor BBB penetration profile. N-acetylcysteine is a precursor of the natural intracellular antioxidant GSH. As a thiol, AD4 may also raise intracellular cysteine, which also acts as an antioxidant. It may stimulate activities of cytosolic enzymes involved in the GSH synthesis cycle. It may inhibit the reactive oxygen species-induced apoptotic pathway as it blocks JNK and p38 mitogen-activated protein kinase phosphorylation.[13–15] It may also protect the mitochondrial respiratory chain proteins from oxidative damage and preserve their bioenergetic capacity. In summary, the novelty of using AD4 over other antioxidants tested so far in the VCM model is that it crosses the BBB, and serves as a carrier for N-acetylcysteine and cysteine into the brain. Because there are no other compounds that share these qualities, it is difficult to determine whether the antioxidative activity of AD4 is the main or the only factor that improves the behavioral changes, or there are other physiologic pathways (ie, interaction with the drug). Future experiments should examine different levels and the superiority of AD4 compared with known antioxidants.

Oxidative stress has been implicated as a major pathogenetic factor in many neurodegenerative disorders including Alzheimer disease and Parkinson disease.[12] It is believed that the accumulation of reactive oxygen species without adequate neutralization by the naturally occurring antioxidant defense system is toxic to neurons and causes either directly, or contributes indirectly, to premature degeneration.[20] Reactive oxygen species increase in the brain during antipsychotic therapy.[21] Exposure to antipsychotics is toxic to cells and induces cell death in vitro via enhanced oxidative stress.[6,22,23] However, it is still unknown by which mechanism oxidative stress induces TD after chronic antipsychotic therapy. TD does not occur in all antipsychotic-treated patients, but only in those with preexisting susceptibility. Furthermore, in many patients, TD may first emerge after discontinuation of these drugs. Therefore, it seems unlikely that the involuntary movements are due to neuronal cell loss in brain. Oxidative stress may somehow act to prime perturbations in dopaminergic receptors and their target circuitries in the basal ganglia induced by antipsychotic therapy in susceptible patients. Our study demonstrated that VCMs induced by haloperidol, an experimental model for TD, were decreased by AD4, the BBB-penetrating antioxidant. These findings lend support to the role of oxidative stress in the development of TD. Furthermore, it indicates that AD4 should be a potential drug to be tested in clinical studies for preventing various side effects in psychiatric patients treated chronically with antipsychotic agents.

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