0026-895X/01/6004-838–846$3.00
MOLECULAR PHARMACOLOGY
Vol. 60, No. 4
884/931571
Mol Pharmacol 60:838–846, 2001
Printed in U.S.A.

Inhibition of Acetylcholine Synthesis and Tyrosine Nitration Induced by Peroxynitrite Are Differentially Prevented by Antioxidants

LYDIE GUERMONPREZ, CLAIRE DUCROCQ, AND YVETTE MOROT GAUDRY-TALARMAIN

Laboratoire de Neurobiologie Cellulaire et Moléculaire (L.G., Y.M.G.-T.), Institut de Chimie des Substances Naturelles (C.D.), Centre National de la Recherche Scientifique, Gif-sur-Yvette, France

Received February 12, 2001; accepted June 29, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Evidence of an overload of reactive oxygen species and peroxynitrite, a derivative of nitric oxide, in sporadic amyotrophic lateral sclerosis suggests that peroxynitrite could impair cholinergic functions. Because of the impossibility of obtaining synaptosomes from vertebrate neuromuscular junctions, we used cholinergic synaptosomes purified from *Torpedo marmorata* electroneurons to characterize the defects triggered by peroxynitrite in more detail. Addition of peroxynitrite or its donor 3-morpholinosydnonimine abolished high-affinity choline uptake and synthesis of acetylcholine from acetate. *T. marmorata* choline acetyltransferase (ChAT) was impaired to the same extent as bovine brain ChAT. A hallmark of peroxynitrite action is the nitration of tyrosine residues in proteins. Peroxynitrite induced a concentration-dependent appearance of nitrotyrosines in several neuronal proteins from synaptosomes and, more readily, from synaptic vesicles. Peroxynitrite also triggered tyrosine nitrations in purified ChAT. Peroxynitrite-dependent nitrations were impaired when synaptosomes were pretreated with thioreductants (glutathione, *N*-acetyl cysteine, dithiothreitol) or antioxidants (uric acid, melatonin, bovine serum albumin, desferrioxamine). Deleterious effects of peroxynitrite on choline transport and ChAT activity were prevented by the thioreductants but only partially by the antioxidants, suggesting a mechanism other than tyrosine nitration, which may involve cysteine oxidation. Further development of protective agents acting on choline transport and on ChAT activity may offer interesting therapeutic possibilities with respect to cholinergic dysfunction occurring in neurodegenerative diseases.

Nitric oxide ($NO^{\cdot}$) modulates neurotransmission by cholinergic neurons (Mothet et al., 1996). At the level of the neuromuscular junction, $NO^{\cdot}$ is produced endogenously, mainly in the skeletal muscle by type-1 $NO^{\cdot}$ synthase (Nakane et al., 1993), where it can interfere with the acetylcholine (ACh) release mechanism after diffusion and participates in the control of muscular force. $NO^{\cdot}$ donors are administered therapeutically to treat various diseases characterized by a deficiency in $NO^{\cdot}$ synthesis. For example, molsidomine, a drug used for the last 30 years, is hydrolyzed to 3-morpholinosydnonimine (SIN-1), which has been shown to release consecutively $O_2^{\cdot-}$ and $NO^{\cdot}$ under oxygenated conditions (Bohn and Schonafinger, 1989; Feelisch et al., 1989). In other pathological conditions or in response to stressful situations, $NO^{\cdot}$ is produced at high concentrations and can spread from cellular compartments. $NO^{\cdot}$ can then combine with $O_2^{\cdot-}$, which is formed at sites of free-radical generation (Beckman et al., 1990; Pryor and Squadrito, 1995) such as mitochondria, or through enzyme activity (e.g., monoamine oxidase, xanthine oxidase), to form peroxynitrite ($ONOO^-$). Recent studies have revealed that $ONOO^-$ is a highly reactive molecule that is able to induce many changes in proteins by oxidizing the sulfhydryl groups of cysteine and methionine as well as tryptophan residues and selectively nitrating tyrosine residues (Pryor and Squadrito, 1995; Radi et al., 2001).

Recently, the detection of nitrotyrosine residues in the brains of patients with Parkinson's disease (Good et al., 1998) and in model animals (Ara et al., 1998) led to a search to determine the molecular targets of $ONOO^-$. Clinical studies showed that tyrosine hydroxylase, the first and rate-limiting enzyme in catecholamine biosynthesis that is selectively affected in Parkinson's disease, is nitrated, suggesting that the cause of the pathology may be an $ONOO^-$ overload (Ara et al., 1998). In cholinergic pathologies, brain areas of patients with Alzheimer's disease showed an increase of $ONOO^-$-mediated nitration of proteins (Smith et al., 1997), and the cerebrospinal fluid of patients with sporadic amyotrophic

This work was supported by the Centre National de la Recherche Scientifique and the Institut de Recherches Internationales Servier.

ABBREVIATIONS: $ONOO^-$, peroxynitrite; ACh, acetylcholine; SIN-1, 3-morpholinosydnonimine; CoA, Coenzyme A; ChAT, choline acetyltransferase; VAMP/synaptobrevin, vesicular-associated membrane protein; TCA, trichloroacetic acid; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; GSH, glutathione; NAC, *N*-acetylcysteine; DTT, dithiothreitol.

lateral sclerosis showed an increase in 3-nitrotyrosine (Beal et al., 1997). Despite the detection of tyrosine nitration in synaptic proteins (Di Stasi et al., 1999; Koppal et al., 1999), no information on peripheral cholinergic proteins is available. Faced with the impossibility of purifying nerve endings of the neuromuscular junction, we investigated the effects of $NO^{\cdot}$ and $ONOO^{-}$ on synaptosomes of a well-characterized model: the electromotoneurons of *Torpedo marmorata*. We demonstrated previously the presence of type-1 $NO^{\cdot}$ synthase in the cell bodies and nerve endings of *T. marmorata* electroneurons and showed that $NO^{\cdot}$ increased ACh release, whereas $ONOO^{-}$ inhibited ACh synthesis (Morot Gaudry-Talarmain et al., 1997).

ACh in neuromuscular junctions is synthesized from two extracellular precursors. The first is acetate, which is converted into acetyl-CoA by acetyl-CoA synthetase. The second is choline, which is internalized by a sodium-dependent and hemicholinium-sensitive transporter (Okuda et al., 2000). Synthesis of ACh from choline and acetyl-CoA is performed by choline acetyltransferase (ChAT), a cytosolic enzyme (Wu and Hersh, 1994). In a subsequent step, newly synthesized ACh is transported into synaptic vesicles by the energy-dependent vesicular ACh transporter.

This article presents the inhibitory effects of $ONOO^{-}$ and SIN-1, a donor of $ONOO^{-}$, on high-affinity choline uptake, ACh synthesis from radiolabeled acetate, and ChAT activity. $ONOO^{-}$-dependent changes in several presynaptic proteins (ChAT, synaptophysin, VAMP/synaptobrevin, actin, tubulin) present in *T. marmorata* synaptosomes and synaptic vesicles were examined and showed examples of nitration of tyrosines and covalent oligomerization. To counteract $ONOO^{-}$ toxicity, several endogenous and exogenous compounds were tested. Antioxidants and thioreductants prevented nitration, but only thioreductants fully protected high-affinity choline uptake and ChAT activity.

Experimental Procedures

Animals

T. marmorata were purchased from the marine station in Roscoff, France, and kept in oxygenated artificial sea water tanks.

Materials

Stock Solutions of ChAT. Bovine brain ChAT (E.C. 2.3.1.6, C-3388 batches 36F9625 and 100H9500) was obtained from Sigma (St. Louis, MO). For each experiment, the lyophilized powder of partially purified ChAT was solubilized in 50 mM sodium-phosphate buffer, pH 7.3 (10 mg/ml or as specified), and used for analysis by Western blotting and measurement of ChAT activity.

Stock Solutions of SIN-1. SIN-1 was obtained from BIOMOL (Plymouth Meeting, PA). Aqueous solutions of SIN-1 (100 mM) were stored at −20°C before use. Monoclonal antibodies against nitrotyrosine, ChAT, and actin (JLA20) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), Chemicon International (Temecula, CA), and DSHB (University of Iowa, Iowa City, IA), respectively. Polyclonal antibody against *T. marmorata* synaptophysin and monoclonal antibodies against VAMP/synaptobrevin and tubulin were developed and characterized in the laboratory by Dr. Nicolas Morel (Laboratoire de Neurobiologie Cellulaire et Moleculaire, CNRS, Gif-sur-Yvette, France). Polyclonal antibody against muscle lactate dehydrogenase was obtained from Chemicon. Other reagents were obtained from commercial sources, and the highest purity available was obtained.

Chemical $ONOO^{-}$ Synthesis

Alkaline $ONOO^{-}$ was synthesized at room temperature using the procedure described by Uppu and Pryor (Uppu and Pryor, 1996) in the two-phase system using isoamyl nitrite and hydrogen peroxide. After a freezing step (−20°C, overnight) of the obtained solution, the upper yellow phase containing concentrated $ONOO^{-}$ (between 1.3 and 2.4 M) was collected. Residual hydrogen peroxide was not removed, and diethylenetriaminepentaacetic acid was added to minimize inadvertent trace-metal contamination. The final stock concentration of this solution was quantified spectrophotometrically after dilution in 0.1 N NaOH at 302 nm (ϵ_M = 1670 $M^{-1} \cdot cm^{-1}$) immediately before each experiment, and appropriate dilutions of $ONOO^{-}$ were performed in 0.1 N NaOH (100 μM to 500 mM $ONOO^{-}$).

Methods

Preparation of Synaptosomes and Synaptic Vesicles. *T. marmorata* electric organ was used to purify cholinergic nerve endings and synaptic vesicles. Synaptosomes were isolated on isoosmotic sucrose-saline Krebs' gradients and were collected at the interface of 0.3 to 0.5 M sucrose according to the method described by Morel et al. (1977). The synaptosomal fraction (40–50 ml) derived from 25 g of electric organ was collected from a gradient made of discontinuous isoosmotic saline-sucrose Krebs' solution devoid of Ca^{2+} and equilibrated to pH 7.4 with 5 mM $NaHCO_3$. Aliquots were used immediately for functional studies. For Western blot analysis of proteins, after treatment with drugs, the synaptosomal suspension was diluted in Krebs' buffer and centrifuged at 12,000*g* for 20 min.

Synaptic vesicles were isolated according to the method described by Israël et al. (1980) and collected at the interface of a 0.38 to 0.5 M sucrose-saline isoosmotic gradient, then further concentrated by centrifugation in 10 mM Tris, 350 mM KCl, and 0.1 M sucrose buffer, pH 7.2.

Exposure of Synaptosomes, Synaptic Vesicles, and Bovine Brain ChAT to $ONOO^{-}$ or SIN-1. Preincubation of synaptosomes (in Krebs' buffer), synaptic vesicles (in Tris buffer), or purified bovine brain ChAT (in 50 mM sodium-phosphate buffer, pH 7.3) with $ONOO^{-}$ or SIN-1 was carried out at room temperature for 1 to 2 h before freezing. For $ONOO^{-}$ and SIN-1 incubations, various increasing concentrations of drugs were added in one bolus directly on the neuronal fractions and stirred immediately, respectively, by a vortex and magnetic agitation in oxygenated *T. marmorata* medium. Usually, $ONOO^{-}$ concentration of stock solutions was 1 to 2 M. $ONOO^{-}$ was then diluted in 0.1 N NaOH and used for physiological studies at final concentrations of less than 4 mM, leading to a minimal dilution (v/v) of 1/250. According to Uppu and Pryor (1996), we estimated that the residual H_2O_2 concentration in the peroxynitrite stock solution may reach 0.2 to 0.5 M, leading to a maximal final concentration of 1 to 2 mM in treated samples.

Using three different types of analysis, we verified that the addition of $ONOO^{-}$ did not induce a significant lysis of synaptosomes. Synaptosomes were treated by increasing concentrations of $ONOO^{-}$ and concentrated by centrifugation. Released protein content after $ONOO^{-}$ treatment was measured in the supernatants: 1) by the Lowry method for total protein; 2) by immunodetection of muscle lactate dehydrogenase after Western blotting; and 3) by the measurement of the volume variations of the synaptosomes. The three different sorts of analysis gave comparable results and confirmed that for up to 500 μM $ONOO^{-}$, there is no lysis of synaptosomes. A 25 to 30% leakage of proteins started to be observed at 1 mM $ONOO^{-}$ (data not shown).

ChAT activity was measured using aliquots of the same synaptosomal samples, and a protein analysis was done on the concentrated preparations. Protein content was determined by the Lowry method of assay.

Synthesis and Compartmentalization of ACh. Synthesis of ACh by 400 μl of synaptosomes was measured using [^{14}C]acetate (100 μM) and choline (100 μM) as ACh precursors. At the end of the

synthesis time (usually 60 min), aliquots (20 μl) were saved and kept frozen for further determination of ChAT activity. Formation of newly synthesized radioactive ACh in synaptosomes (180 μl) was stopped by the addition of 5% trichloroacetic acid (TCA), whereas in the other aliquot (180 μl), radioactive ACh accumulation in the vesicular pool was determined after one freezing and thawing cycle of the synaptosomes (Dolezal et al., 1993), followed by 5% TCA addition. After extraction on ice, TCA was eliminated from all the samples by three ether washes, and radioactive ACh was extracted by an allylcyanide-tetraphenylboron organic extraction procedure (Fonnum, 1975). The organic lipophilic phase containing radioactive ACh was collected, and radioactivity was counted in Lipoluma scintillation liquid (Lumac, Landgraaf, the Netherlands).

ChAT Assay. ChAT activity was determined using 10-μl aliquots. The treated enzyme and synaptosomes were collected at the end of the incubation and frozen until the assay. Samples were warmed to room temperature and mixed with detergent [0.02% Triton X-100 (w/v)] for 1 min to disrupt membranes. The reaction was started by the addition of the substrates [1-^{14}C]acetyl-CoA (14 μM) and choline (2.5 mM) in the presence of 250 μM eserine, 10 μM bovine serum albumin (BSA), and 75 mM NaCl and stopped after 1 h by dilution with cold sodium-phosphate buffer. Radioactive ACh was extracted and determined according to the method used by Fonnum (1975). Typical ChAT activity of control samples was 207.7 $\pm$ 24.1 nmol $\cdot$ h^{-1} $\cdot$ g^{-1}, in accordance with the findings of Morel et al. (1977).

High-Affinity Choline Uptake Assay. Choline accumulation in nerve endings was measured by use of the following method: after 15 min of rewarming at room temperature, freshly prepared synaptosomes (300 μl) were pretreated with ONOO$^-$, SIN-1, or vehicle (0.1 N NaOH) for 45 min. High-affinity choline uptake was initiated by the addition of [^{14}C]choline (5 μM) and terminated by filtration on a 1.2-μm filter (Millipore Corporation, Bedford, MA). After subsequent washing of the filter with 10 ml of cold physiological medium, pH 7.4, the incorporated [^{14}C]choline was determined by liquid scintillation counting.

SDS-PAGE Analysis. ONOO$^-$-treated samples (synaptosomes or purified enzymes) were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions (β-mercaptoethanol 10% in lysis buffer) followed by Western blotting onto nitrocellulose. Western blots were probed with primary antibodies for 3 to 12 h at room temperature. Probed proteins were detected by secondary antibodies linked to horseradish peroxidase and visualized by the enhanced chemiluminescence kit (ECL; Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Results

Exogenous ONOO$^-$ or SIN-1 Inhibits High-Affinity Choline Uptake and [^{14}C]ACh Synthesis from [^{14}C]Acetate. As shown in Fig. 1, increasing concentrations of ONOO$^-$ (Fig. 1A) or SIN-1 (Fig. 1B) in the preincubation medium of synaptosomes resulted in a dose-dependent inhibition of high-affinity choline uptake. An inhibition of 50% was obtained at approximately 500 μM for ONOO$^-$ and 800 μM for SIN-1. After pretreatment with 1 mM ONOO$^-$, choline uptake was nearly totally abolished. H_2O_2 was without effect (Fig. 1C), even in the millimolar range, showing that ONOO$^-$ effects were not related to residual traces of H_2O_2 present in the preparation of ONOO$^-$ (Uppu and Pryor, 1996).

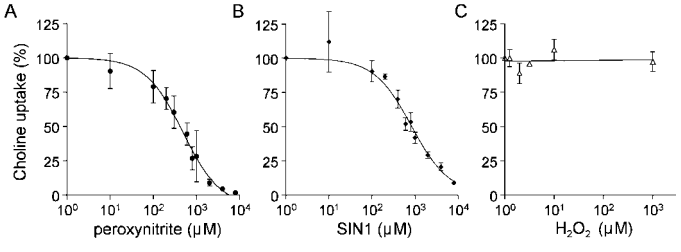

Fig. 1. ONOO$^-$ and SIN-1 but not H_2O_2 inhibit high-affinity choline uptake. Synaptosomes were preincubated with various concentrations (10 μM to 8 mM) of ONOO$^-$ (A), SIN-1 (B), or H_2O_2 (C) for 45 min before the addition of 5 μM [^{14}C]choline. Data shown are mean $\pm$ S.E.M. from values obtained for three different preparations and normalized to values relative to control (vehicle-treated) preparations.

As shown in Fig. 2, increasing concentrations of ONOO$^-$ dose-dependently inhibited [^{14}C]ACh synthesis (IC_{50} = 300 μM; Fig. 2A) but not its incorporation into vesicles (Fig. 2B). Continuous infusion with SIN-1 (Fig. 2A′, 2B′) led to similar results, with a lesser potency (IC_{50} = 900 μM). H_2O_2 (200 μM) did not induce any significant changes in the synthesis and incorporation of ACh (data not shown).

ONOO$^-$ Inhibits ChAT Activity. Having shown that ACh synthesis from acetate was defective in ONOO$^-$-treated synaptosomes, we wanted to establish whether the enzymatic activity of ChAT, the enzyme responsible for ACh synthesis from acetyl-CoA and choline, was altered, or if this inhibition was subsequent to the inactivation of choline uptake, which provides the other ACh precursor, choline. Therefore, synaptosomes pretreated with ONOO$^-$ or SIN-1 were lysed by Triton X-100 (0.02%) and assessed for the activity of intracellular ChAT at the end of the measurement of the ACh synthesis. The enzyme activity was inhibited by ONOO$^-$ (IC_{50} = 350 μM) and by SIN-1 (IC_{50} = 40 μM) (Fig. 2, C and C′). SIN-1 was surprisingly a more potent inhibitor of ChAT activity of synaptosomes than ONOO$^-$. ChAT activity was determined after solubilization of the synaptosomal membrane with Triton X-100 and dilution of the cytosol, which leads to better oxygen accessibility during SIN-1 decomposition. Moreover, SIN-1 decomposition in tissues can lead to the continuous formation of oxidized toxic substances

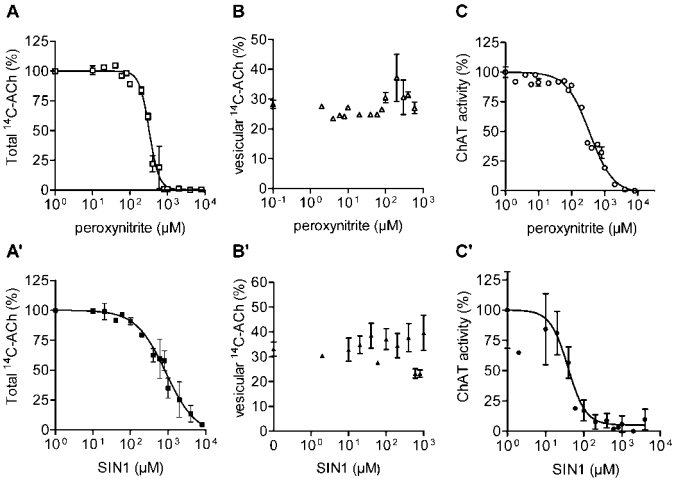

Fig. 2. ONOO$^-$ and SIN-1 inhibit ACh synthesis and ChAT activity but not compartmentalization of ACh into synaptic vesicles. Presented results obtained from samples of three to six different preparations are the effects of a 60-min preincubation of synaptosomes with either chemically synthesized ONOO$^-$ (open symbols) or SIN-1 (filled symbols). A, A′, total [^{14}C]ACh synthesis measured for 60 min after treatment using [^{14}C]acetate as a radioactive precursor and expressed as mean $\pm$ S.E.M. of a percentage of controls. B and B′, incorporation of [^{14}C]ACh in synaptic vesicles measured for the same time after a cycle of freezing/thawing and expressed as a percentage of total newly synthesized [^{14}C]ACh. C and C′, ChAT activity measured using [1-^{14}C]acetyl-CoA as a substrate after Triton X-100 solubilization of membranes and expressed as mean $\pm$ S.E.M. of a percentage of controls.

such as peroxidized fatty acids and proteins (Koppal et al., 1999; Tien et al., 1999), which might interfere with the measurement of enzyme activity (Currier and Mautner, 1976).

To determine whether the effects of $ONOO^-$ on ChAT depended on its subcellular distribution in *T. marmorata* motoneurons, we compared the activity of synaptosomal ChAT with that contained in the cell bodies concentrated in the electric lobe (data not shown). ChAT activity was inhibited by $ONOO^-$ in both structures with comparable IC_{50} values (400 μM, $n = 8$; and 300 μM, $n = 3$, respectively). We showed that 1 mM H_2O_2 was without effect on the ChAT activity of synaptosomes (100.96 ± 1.55% of untreated control, $n = 3$).

As in the previous experiments performed with *T. marmorata* nerve endings showing an inhibition of the activity of ChAT by $ONOO^-$ and SIN-1, we investigated the effect of $ONOO^-$ and SIN-1 on partially purified bovine brain ChAT in the presence of Triton X-100 (0.02%) in sodium-phosphate buffer, pH 7.3. ChAT activity was inhibited by $ONOO^-$ and, to a lesser extent, by SIN-1, possibly because of a lack of oxygen in the medium (Fig. 3). An inhibition of 50% was obtained with 300 μM $ONOO^-$ and was total at concentrations of nearly 1 mM $ONOO^-$. This result shows that $ONOO^-$ probably directly modifies the ChAT protein itself.

Nitrotyrosine Detection in Purified ChAT after $ONOO^-$ Treatment. Nitration of tyrosine residues in proteins is an important post-translational modification triggered by $ONOO^-$. Therefore, we sought the presence of nitrotyrosine in untreated bovine brain ChAT and in $ONOO^-$-treated samples by Western blot analysis.

Coomassie blue staining (Fig. 4A, control lane) after reducing SDS-PAGE shows that the commercial ChAT preparation (batch 36F9625, 10 mg/ml, 5 μl per lane) is mainly constituted by one major band at 63 kD. However, two bands at 65 and 67 kDa are also clearly revealed by the anti-ChAT antibody (Fig. 4C). Despite the fact that the protein content was the same in every sample before $ONOO^-$ treatment, a decrease of the Coomassie blue staining was noticed after treatment of ChAT at the highest $ONOO^-$ concentrations (Fig. 4A), suggesting possible degradation of the protein. Likewise, the immunoreactivity of the 65- and 67-kDa bands decreases at concentrations of $ONOO^-$ greater than 200 μM (Fig. 4C).

Figure 4B shows that no nitrotyrosine labeling occurs in the controls or at low concentrations of $ONOO^-$ (10 μM). At 100 μM $ONOO^-$, the 63-kDa band is labeled, as well as another band at 61 kDa that was not detected otherwise. At concentrations greater than 100 μM $ONOO^-$, we showed a concentration-dependent increase of the chemiluminescent signal that became saturated at 1 mM $ONOO^-$. At high concentrations of $ONOO^-$, other bands have nitrotyrosine immunoreactivity at higher and lower molecular masses. This suggests that a possible oligomerization of ChAT (by di-tyrosine cross-linking, for example) and lysis may occur. We were unable to detect nitration by SIN-1, and no significant change in the immunoreactivity for ChAT was noticed after SIN-1 treatment.

Nitrotyrosine Appearance in Presynaptic Proteins after $ONOO^-$ Treatment Is Prevented by Thioreductants and Antioxidants. Analysis of the possible changes triggered by an $ONOO^-$ attack on neuronal proteins was further pursued by tracking down nitrotyrosines or other modifications on synaptic proteins after treatment with $ONOO^-$. Therefore, we treated a synaptic vesicles preparation (Fig. 5) or intact, functional synaptosomes (Fig. 6) with increasing $ONOO^-$ concentrations.

Isolated vesicles were more sensitive to $ONOO^-$ than the synaptosomal fraction. In synaptic vesicles, the nitrotyrosine immunoreactivity was observed at low concentrations of $ONOO^-$ (100 μM) and, even at higher $ONOO^-$ concentrations, was specific to certain proteins because VAMP/synaptobrevin, for example, was not nitrated (Fig. 5, A and B). Among the nitrated proteins, two proteins migrating at molecular masses of 43 kDa and 52 kDa seemed to be nitrated early. They comigrate with actin (Fig. 5C) and tubulin (Fig. 5D), respectively. Immunoreactivity against tubulin was revealed by an antibody developed against *T. marmorata* tubulin, which recognizes tubulin at 50 kDa and another uncharacterized ≈40-kDa band specific for *T. marmorata* nerve terminals (Sbia et al., 1991). When blots were stripped and reprobed with antiactin and antitubulin antibodies (Fig. 5, C and D, respectively), we observed the coincident disappearance of their immunoreactivity with the appearance of nitrotyrosines.

Synaptophysin, an integral vesicular 38-kDa glycoprotein, was detected in the controls (Fig. 5E) as a doublet using an antibody directed against the C-terminal epitope ^{265}GYQPNYGQ^{277}Q (Cowan et al., 1990; N. Morel, personal communication). We found that its immunoreactivity was lost upon treatment with $ONOO^-$.

VAMP/synaptobrevin was not nitrated after $ONOO^-$ action, and it did not lose its immunoreactivity after peroxynitrite action. Furthermore, we observed that the immunoreactivity of a 30- to 32-kDa VAMP-containing complex resistant to SDS, β-mercaptoethanol, and boiling increased after $ONOO^-$ treatment of synaptic vesicles (Fig. 5A). In contrast, the B-regulatory subunit of calcineurin, which is present in the synaptic vesicles preparation, is not nitrated by peroxynitrite and nevertheless loses its immunoreactivity after peroxynitrite action (not shown).

By comparison with isolated synaptic vesicles, only a few proteins, yet uncharacterized, were sensitive to 1 mM $ONOO^-$ when functional synaptosomes were treated with increasing concentrations of $ONOO^-$ (Fig. 6). Among these proteins, the faint 63-kDa nitrated protein may be ChAT. As in synaptic vesicles, no nitration occurs for VAMP/synaptobrevin, but oligomerization was observed (data not shown).

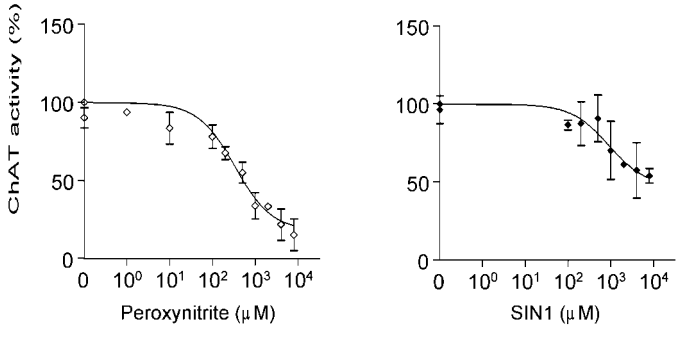

Fig. 3. $ONOO^-$ and SIN-1 inhibit purified bovine brain ChAT. After a 60-min preincubation with $ONOO^-$ or SIN-1, the activity of 10 μl of bovine brain ChAT diluted (10 mg/ml) in 50 mM sodium phosphate buffer, pH 7.3,was measured using [1-^{14}C]acetyl-CoA as a substrate. Results are expressed as mean ± S.E.M. of a percentage of controls (obtained from three different commercial preparations of enzyme).

When synaptosomes were treated with increasing concentrations of SIN-1 (100–4000 μM) for 1 h under continuous agitation and ambient aerobic conditions, no immunoreactivity for nitrotyrosine was detected, and only faint bands appeared at 4 mM SIN-1 (data not shown).

As shown for tryptophan hydroxylase (Kuhn and Geddes, 1999), protection against $ONOO^-$-induced tyrosine nitrations may prevent the loss of enzymatic activity. To assess further the interrelationship between the nitration of synaptosomal proteins induced by $ONOO^-$ and the inhibition of cholinergic functions, we tried to prevent the damage or to intercept the oxidizing and nitrating reactive species, as suggested previously (Arteel et al., 1999; Blanchard et al., 2000). Synaptosomes were pretreated with the following thioreduc-

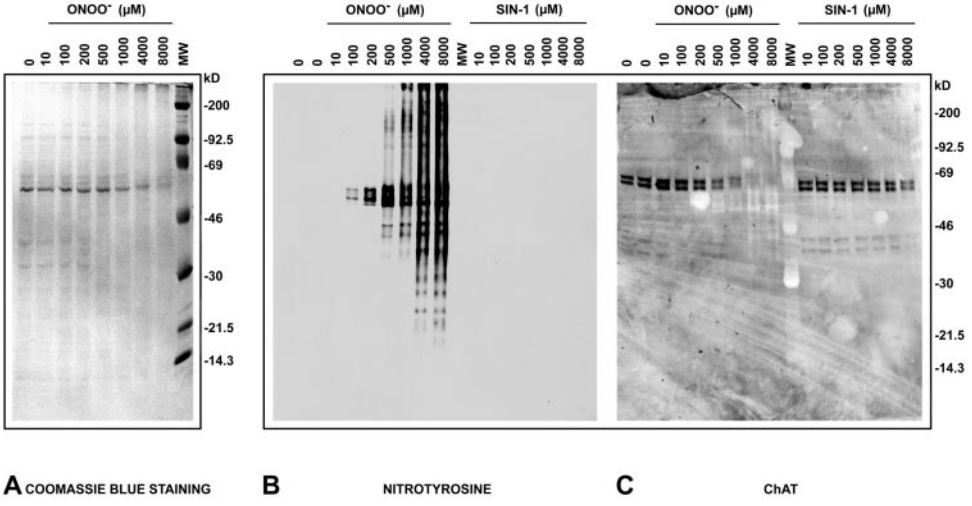

Fig. 4. Bovine brain ChAT is nitrated by $ONOO^-$. Partially purified bovine brain ChAT (Sigma, N°C 3388) diluted in sodium-phosphate buffer, pH 7.3 (10 mg/ml), was nitrated by increasing concentrations of $ONOO^-$ (10–8000 μM) or treated similarly with water or the vehicle 0.1 N NaOH (0) at room temperature for 2 h and transferred to the cold room overnight before SDS-PAGE analysis. Samples (5 μl) were analyzed by Western blotting using 5 to 15% gradient gels under reducing conditions (10% β-mercaptoethanol) and after boiling for 5 min. Presented results are typical of five different experiments. A, Coomassie blue staining of the preparation. B, Western blot of nitrated ChAT was analyzed using monoclonal antibody raised against nitrotyrosine (1/1000) and revealed by ECL-peroxidase system. C, after stripping the immunolabeling for nitrotyrosine, washing, and saturating in 3% milk in Tris-buffered saline, the blot was incubated with polyclonal antibody against ChAT (1/500). The immunoreactivity for ChAT was similarly revealed by ECL-peroxidase system.

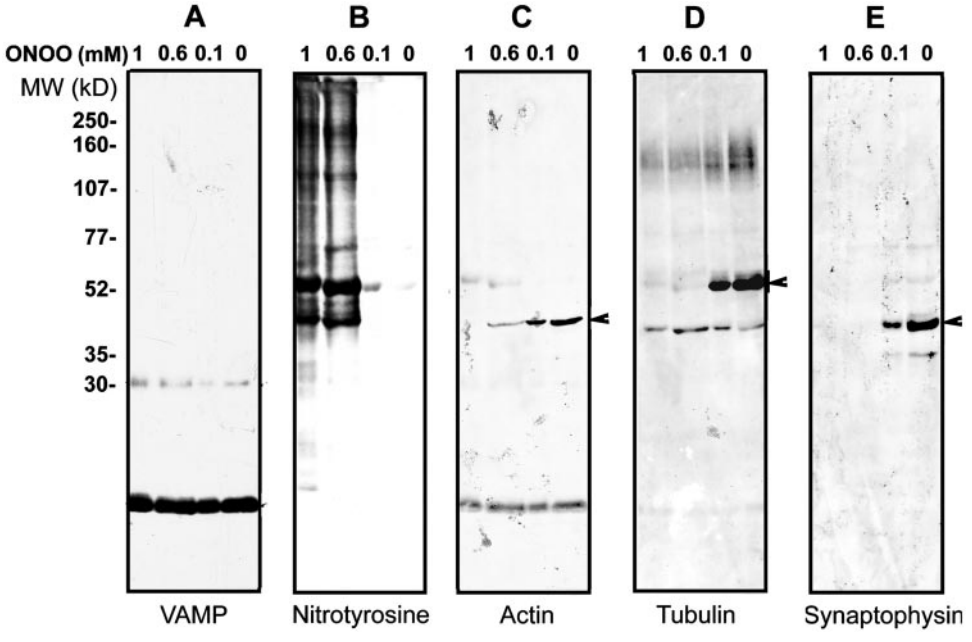

Fig. 5. $ONOO^-$ is a powerful nitrating agent on proteins of synaptic vesicles. Four different preparations of synaptic vesicles were used to test the effect of increasing concentrations of $ONOO^-$. Results of a typical experiment are presented. Synaptic vesicles were treated (100 min) with $ONOO^-$ from 0 to 1 mM at room temperature and concentrated by centrifugation. Pellets were suspended in lysis buffer in the presence of 10% β-mercaptoethanol and boiled for 5 min, and aliquots (100-μg proteins) were analyzed. B, immunolabeling for monoclonal antinitrotyrosine (1/1000). After stripping the antinitrotyrosine antibody from the blot, washing, and saturating in 3% milk in Tris-buffered saline, the blot was incubated with different antibodies against neuronal proteins and revealed by ECL-peroxidase system. Arrows show actin (1/200, C), tubulin (1/1000, D), synaptophysin (1/2500, E), and VAMP/synaptobrevin (1/25, A). Note that actin was immunodetected after stripping the anti-VAMP/synaptobrevin antibody, which explains residual labeling at 18 kDa (C). Also, the antibody developed against *T. marmorata* tubulin (Sbia et al., 1991) recognizes tubulin at 50 kDa and another uncharacterized protein (≈40-kDa band) that is insensitive to $ONOO^-$ (D).

tants: 5 mM glutathione (GSH), 1.25 mM *N*-acetylcysteine (NAC), and 1 mM dithiothreitol (DTT); and with the following antioxidants: 1 mM uric acid, 1 mM melatonin, 0.2 mM desferrioxamine, and 1 mM BSA, for 30 min before the addition of 1 mM $ONOO^-$. In Fig. 6, it can be seen that all these compounds were able to prevent effectively the appearance of nitrotyrosine in synaptosomes.

Thioreductants and Uric Acid Protect Choline Uptake. In further studies, we changed the synaptosomal oxidation state by pretreatment with the same thioreductants (GSH, NAC, DTT) and antioxidants (uric acid, BSA, desferrioxamine, melatonin). Apart from BSA, which increased choline uptake, these compounds did not affect the basal rate of choline transport. We show in Fig. 7A that all thioreductants, uric acid, and BSA significantly protected choline uptake from $ONOO^-$ inhibition. Surprisingly, desferrioxamine and melatonin did not affect choline transport, but they prevented all the nitration reactions.

Thioreductants Fully Protect and Partially Restore ChAT Activity. Similarly, we tested the protective properties of thioreductants and antioxidants against the loss of ChAT activity of synaptosomes caused by $ONOO^-$. Although the thioreductants fully protected ChAT activity (Fig. 7B), we did not observe protection with any of the other antioxidant compounds tested (uric acid, BSA, desferrioxamine, melatonin).

As demonstrated previously (Viner et al., 1999; Radi et al., 2001), formation of disulfides or nitrosothiol products by $ONOO^-$ is reversible by thiol-reducing agents. The reversibility of $ONOO^-$ action on ChAT activity was assessed by treating synaptosomes first with $ONOO^-$ and then with DTT (5 mM). As shown in Fig. 8, DTT was able to significantly improve ChAT activity after 200 μM and 1 mM $ONOO^-$ action.

Discussion

The pathological production of $ONOO^-$ at the presynaptic side of neuromuscular junctions was mimicked in vitro using either a dose of the chemically synthesized $ONOO^-$ or a donor, SIN-1, which released $ONOO^-$ by a regular flux associated with molecular degradation. In physiological buffers containing $NaHCO_3$, $ONOO^-$ is transformed into the species nitroso-peroxicarbonate ($ONOOCO_2^-$) (Lymar et al., 1996). Both $ONOO^-$ and $ONOOCO_2^-$ decompose into mineral nitrate and nitrite and in the presence of reactive molecules, they are powerful nitrating and oxidizing agents. However, the rapid decay of $ONOO^-$ added extracellularly explains why high doses of $ONOO^-$ (up to 1 mM) are required to affect intracellular targets.

The present results show that under physiological conditions of pH and bicarbonate concentrations, $ONOO^-$ is a powerful inhibitor of two major cholinergic processes: choline transport and ACh synthesis. Furthermore, the analysis by Western blotting of presynaptic proteins, which are modified by $ONOO^-$ (either by nitration of tyrosines, loss of immunoreactivity, or cross-linking), support the idea that it may induce severe alterations of the cholinergic functions.

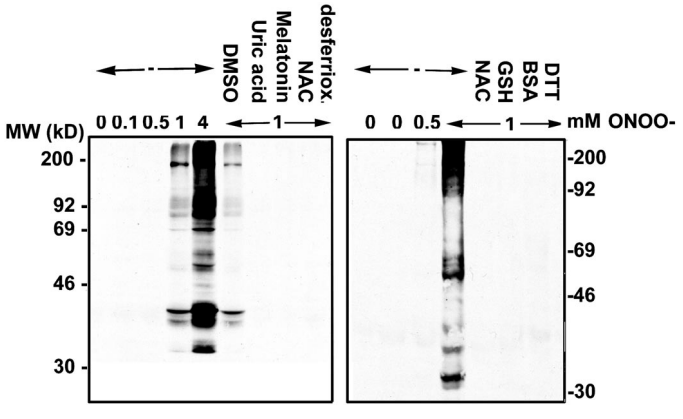

Fig. 6. Tyrosine nitration of synaptosomal proteins by $ONOO^-$ and its prevention by various antioxidants and thioreductants. Results from two different among at least three independent experiments for each compound are presented. Synaptosomes were incubated with various agents for 30 min before the addition of $ONOO^-$ (0–4 mM), which was incubated for 60 min at room temperature. SDS-PAGE analysis of concentrated samples using antinitrotyrosine antibody (1/1000) was performed. Concentration of the drugs were as follows: 5 mM GSH, 1.25 mM NAC, 1 mM uric acid, 1 mM BSA, 1 mM DTT, 200 μM desferrioxamine (deferriox), and 1 mM melatonin.

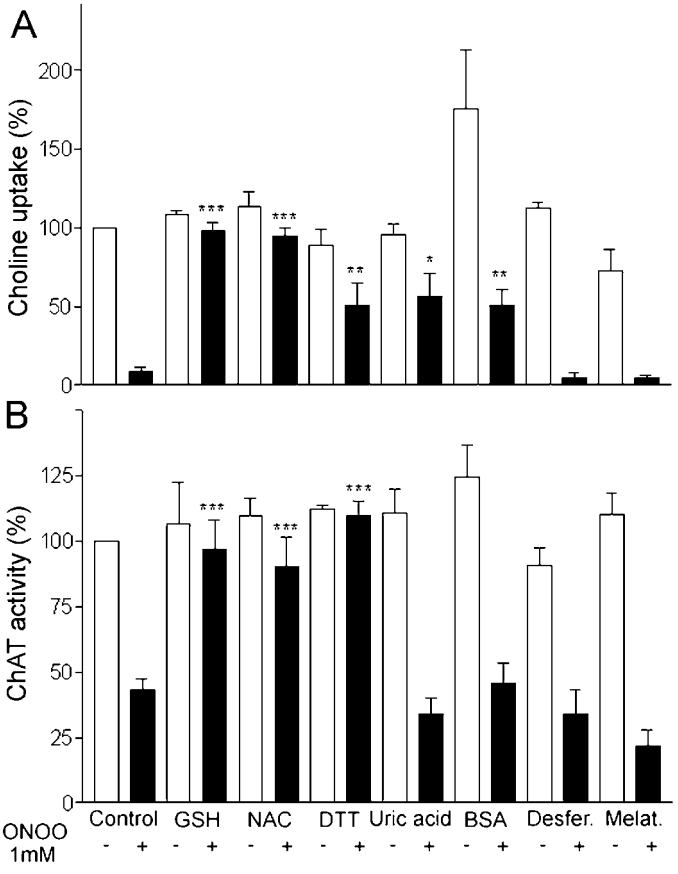

Fig. 7. Protection of high-affinity choline uptake and ChAT activity from $ONOO^-$-induced inactivation. Synaptosomes were incubated with various agents for 30 min before the addition of $ONOO^-$ (1 mM) at room temperature. High-affinity choline uptake measurement and ChAT activity assay were performed after 60 min of $ONOO^-$ treatment. The results represent the mean ± S.E.M. of values obtained from three to seven different experiments (assayed in duplicate) in the presence of protector only or in the presence of both protector and $ONOO^-$ and expressed as a percentage of control values obtained without any treatment. Concentrations of protectors were for the high-affinity choline uptake protection assay (A): 5 mM GSH, 1 mM NAC, 1 mM DTT, 0.5 mM uric acid, 1 mM BSA, 200 μM desferrioxamine (Desfer.), 1 mM melatonin (Melat.); and for ChAT activity protection assay (B): 5 mM GSH, 5 mM NAC, 1 mM DTT, 1 mM uric acid, 1 mM BSA, 200 μM desferrioxamine, 1 mM melatonin.

In cholinergic neurons, the synthesis of ACh by ChAT is dependent on intracellular pools of choline provided by the high-affinity choline uptake and of acetyl-CoA replenished by metabolism. Very recently, the cholinergic-specific high-affinity choline transporter was cloned (Okuda et al., 2000). High-affinity choline uptake, which is present in all cholinergic neurons, is Na^+- and Cl^--dependent, uses the Na^+/K^+-ATPase-maintained membrane potential as a driving force, and is electrogenic (O'Regan, 1988). In this report, choline transport was found to be inactivated in a concentration-dependent manner both by $ONOO^-$ and SIN-1, but not by millimolar ranges of H_2O_2. We did not establish whether this inhibition is direct: it could also be mediated by perturbations of ionic gradients because Na^+/K^+-ATPase was previously shown to be inhibited by $NO^{\cdot}$-donors, $ONOO^-$, and other oxidants (Muriel and Sandoval, 2000). Previous reports showed that liposome-reconstituted Na^+-dependent high-affinity glutamate transport was inhibited by similar doses of $ONOO^-$, but also by high doses (more than 1 mM) of H_2O_2 (Trotti et al., 1996). These effects, reversed by disulfide-reducing agents such as DTT, were attributed to cysteine oxidation. Our results show that selective modifications of choline transport are triggered by $ONOO^-$ and not by the other oxidant H_2O_2 and that these modifications could be prevented by thiols and uric acid. $ONOO^-$ is a stronger oxidant than H_2O_2 and may induce cysteine oxidation beyond disulfide (e.g., into sulfenic, sulfinic, or sulfonic acid) or may oxidize other residues (methionine and tryptophan). It can also trigger tyrosine nitrations. The fact that some compounds (e.g., melatonin or desferrioxamine) that were fully protective for $ONOO^-$-induced nitration were ineffective in protecting choline uptake suggests that tyrosine nitration is not the mechanism that explains selective $ONOO^-$ toxicity toward choline uptake.

ACh synthesis was inhibited by either $ONOO^-$ or SIN-1, but neither was inhibited by H_2O_2 up to 1 mM (this study) or by similar concentrations of the $NO^{\cdot}$ donors *S*-nitroso-*N*-acetylpenicillamine and sodium nitroprusside (Morot Gaudry-Talarmain et al., 1997). To determine whether ChAT itself was affected or whether ACh synthesis inhibition was caused by the prevention of choline uptake, we tested the effect of $ONOO^-$ on *T. marmorata* synaptosomal ChAT or on partially purified bovine brain ChAT. In both preparations, $ONOO^-$ totally inhibited ACh synthesis ($IC_{50} \approx 500\ \mu M$), confirming that both ChAT from mammalian central nervous system and from *T. marmorata* peripheral motoneurons were direct targets of $ONOO^-$. Moreover, we have shown that the inhibition of ChAT by $ONOO^-$ coincides with the appearance of nitrotyrosine and starts at concentrations as low as 50 to 100 μM $ONOO^-$. Hersh et al. (1984) showed previously that several variants of ChAT were present in partially purified bovine brain ChAT preparations differing in their isoelectric points, molecular masses, and affinities for specific antibodies, but not in their enzymatic activities. The pattern of immunoreactivity of bovine brain ChAT after $ONOO^-$ treatment is complex. We showed that the anti-ChAT signal for the 65-kDa and 67-kDa bands decreased as the antinitrotyrosine immunoreactivity of the 63-kDa band increased, leading us to question whether there was a preferential sensitivity of the 63-kDa band of ChAT to nitration of tyrosine. Our work indicates that variants of ChAT may differ in their sensitivity to action of $ONOO^-$. At concentrations of $ONOO^-$ higher than 200 μM, several protein bands presented nitrotyrosine immunoreactivity with higher and lower molecular masses. They were not characterized, but we cannot eliminate the possibility that oligomerization of ChAT, by dityrosine cross-linking, for example, as described previously for α-synuclein by Souza et al. (2000), or proteolysis may occur, requiring further studies.

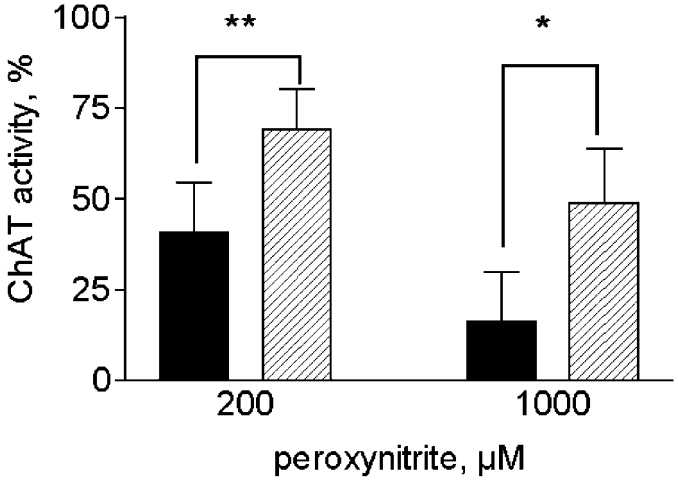

Fig. 8. Partial restoration of $ONOO^-$-inhibited ChAT activity by DTT. Synaptosomes were incubated with $ONOO^-$ (200 μM or 1 mM) during 40 min at room temperature. Then, DTT (5 mM) was added, and ChAT activity assay was performed after 30 min of DTT treatment. The results represent the mean ± S.E.M. of values obtained in duplicates for $ONOO^-$-treated samples from four different experiments, either in the presence (▨) or in the absence (■) of DTT, and expressed as a percentage of control values obtained without $ONOO^-$.

The complex regulation of ChAT activity has been reported previously. The state of phosphorylation, controlled proteolysis, and subcellular localization (cytosolic versus membrane-bound isoforms) and the presence of thiol agents can all modulate the activity of the enzyme posttranslationally (Oda, 1999; Wu and Hersh, 1994). Tyrosine nitration could interfere with some of these pathways. As proposed previously (Beckman and Koppenol, 1996; Di Stasi et al., 1999), there is a modulation of tyrosine-dependent signaling in motoneurons when tyrosines are nitrated. ChAT is phosphorylated by several serine/threonine kinases (protein kinase C, casein kinase II, protein kinase G, and α-calcium/calmodulin-dependent protein kinase II) (Bruce and Hersh, 1989; Dobransky et al., 2000). There is only one conserved tyrosine phosphorylation consensus site on human ChAT, without experimental evidence of its functionality.

$ONOO^-$ is also a potent oxidant known to react with cysteine residues (Viner et al., 1999; Radi et al., 2001). Human ChAT isoforms contain 20 or 24 cysteines, and thiol reagents are known to affect the activity of the enzyme (Currier and Mautner, 1976). Our data are in accordance with these observations: $ONOO^-$ blocks the activity of the enzyme, and the deleterious effect of $ONOO^-$ can be prevented by agents that maintain a high content of free reducing compounds (NAC, GSH, DTT). We did not measure the variations of GSH content in synaptic vesicles and synaptosomes in the presence of $ONOO^-$ and SIN-1. Nevertheless, according to many reports in the literature and data obtained from brain synaptosomes (Koppal et al., 1999), we suspect that a decrease of reducing equivalents and the formation of S-S bonds between cysteines may occur after $ONOO^-$ treatment of synapto-

somes. The fact that $ONOO^-$ inhibition can be at least partially reversed by DTT favors this hypothesis, but the possibility of other DTT-reversible cysteine modifications such as *S*-nitrosylation awaits further investigation. The protective role of thioreductant agents on ChAT activity can be explained by a maintaining action on neuronal GSH pools and on critical reduced cysteines close to the active site of the enzyme. Functional analysis of conserved histidines in ChAT by site-directed analysis revealed their essential role in catalysis as an acid/base sensor (Wu and Hersh, 1994). In this article, we suggest that tyrosines and cysteines may be redox sensors for ChAT. Inactivation by $ONOO^-$ of the enzyme activity by sulfhydryl oxidation was recently shown to be essential for tryptophan hydroxylase, another neurotransmitter synthesis enzyme (Kuhn and Geddes, 1999). Nevertheless, we do not know the impact of the nitration of tyrosines that we observed.

To complete our functional studies, we undertook biochemical characterization of proteins that were affected by $ONOO^-$ through nitration of tyrosines or other modifications. We observed that after purification, the synaptic vesicles devoid of the protection afforded by endogenous reducing compounds present in the cytosol are very sensitive to $ONOO^-$-mediated nitration of tyrosines. We confirmed in peripheral nerve endings results previously obtained in studies of mammalian cells or brain extracts (Beckman and Koppenol, 1996; Eiserich et al., 1999) showing nitrotyrosine immunoreactivity at the molecular masses of tubulin and actin. We showed that after $ONOO^-$ attack of the proteins, the monoclonal antitubulin and antiactin antibodies no longer recognized their targets. This coincident loss of recognition of the epitopes may be caused by changes in the protein structure. As proposed by Eiserich and colleagues, these changes could compromise the function of these proteins and interfere with their binding properties, e.g., with dynein for tubulin (Eiserich et al., 1999). Another protein that was affected was synaptophysin/p38. Thus, we have confirmed with peripheral cholinergic synaptic vesicles the results that Di Stasi et al. obtained using brain synaptosomes (Di Stasi et al., 1999). Moreover, we were able to define affected residues by taking advantage of a specific antibody raised against a well-conserved sequence (^{265}GYQPNYGQ^{273}Q) of the *T. marmorata* synaptophysin (Cowan et al., 1990). This epitope is located at the C terminus of the protein (Cowan et al., 1990) and faces the cytosol of synaptosomes. We showed that $ONOO^-$ (up to 500 μM) induced the loss of recognition by this antibody, indicating that the conserved ^{266}Y and ^{270}Y could be potential targets. We also observed that VAMP/synaptobrevin was not nitrated on tyrosines but formed at 32 kDa a covalent complex that may be the dimer previously obtained with the use of cross-linking agents (Laage and Langosch, 1997).

In summary, we demonstrated that $ONOO^-$ can affect numerous proteins at the presynaptic side of a neuromuscular junction in several subcellular compartments; these include choline transporter at the plasma membrane, ChAT in the cytosol, and synaptophysin, tubulin, or actin at the periphery of synaptic vesicles. $ONOO^-$ can act by various mechanisms including cross-link formation, tyrosine nitration, cysteine oxidation or *S*-nitrosylation that are differentially protected by various antioxidants. These results can be of potential interest for therapeutic research on $ONOO^-$-related neuronal diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, or Parkinson's disease.

Acknowledgments

We thank Dr. Maurice Israël and Dr. Pierre Potier, in whose laboratories this work was performed, for continuous support; Dr. Nicolas Morel for help on immunological studies and the kind gift of antibodies against VAMP/synaptobrevin, synaptophysin, and tubulin; Dr. François-Marie Meunier for sequence alignments; Dr. Seana O'Regan for expertise in choline uptake mechanisms and critical reading; and Dr. Michael Spedding and Dr. Esther Schenker from the Institut de Recherches Internationales Servier for their valuable advices. We are grateful to Dr. Peter Lea for reading and helping with the manuscript, Dr. Jacques Stinnakre for help with software, Jean-Baptiste Milandri for measurement of synaptosomal volume under $ONOO^-$ treatment, and Jean-Paul Bouillot for photography.

References

Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J, and Ischiropoulos H (1998) Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc Natl Acad Sci USA* **95:**7659–7663.
Arteel GE, Briviba K, and Sies H (1999) Protection against peroxynitrite. *FEBS Lett* **445:**226–230.
Beal MF, Ferrante RJ, Browne SE, Matthews RT, Kowall NW, and Brown RH (1997) Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Ann Neurol* **42:**644–654.
Beckman JS, Beckman TW, Chen J, Marshall PA, and Freeman BA (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* **87:**1620–1624.
Beckman JS and Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* **271:**C1424–C1437.
Blanchard B, Pompon D, and Ducrocq C (2000) Nitrosation of melatonin by nitric oxide and peroxynitrite. *J Pineal Res* **29:**184–192.
Bohn H and Schonafinger K (1989) Oxygen and oxidation promote the release of nitric oxide from sydnonimines. *J Cardiovasc Pharmacol* **14 (Suppl 11):**S6–S12.
Bruce G and Hersh LB (1989) The phosphorylation of choline acetyltransferase. *Neurochem Res* **14:**613–620.
Cowan D, Linial M, and Scheller RH (1990) *Torpedo* synaptophysin: evolution of a synaptic vesicle protein. *Brain Res* **509:**1–7.
Currier SF and Mautner HG (1976) Evidence for a thiol reagent inhibiting choline acetyltransferase by reacting with the thiol group of coenzyme A forming a potent inhibitor. *Biochem Biophys Res Commun* **69:**431–436.
Di Stasi AM, Mallozzi C, Macchia G, Petrucci TC, and Minetti M (1999) Peroxynitrite induces tyrosine nitration and modulates tyrosine phosphorylation of synaptic proteins. *J Neurochem* **73:**727–735.
Dobransky T, Davis WL, Xiao GH, and Rylett RJ (2000) Expression, purification and characterization of recombinant human choline acetyltransferase: phosphorylation of the enzyme regulates catalytic activity. *Biochem J* **349:**141–151.
Dolezal V, Sbia M, Diebler MF, Varoqui H, and Morel N (1993) Effect of N,N′-dicyclohexylcarbodiimide on compartmentation and release of newly synthesized and preformed acetylcholine in *Torpedo* synaptosomes. *J Neurochem* **61:**1454–1460.
Eiserich JP, Estevez AG, Bamberg TV, Ye YZ, Chumley PH, Beckman JS, and Freeman BA (1999) Microtubule dysfunction by posttranslational nitrotyrosination of alpha-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc Natl Acad Sci USA* **96:**6365–6370.
Feelisch M, Ostrowski J, and Noack E (1989) On the mechanism of NO release from sydnonimines. *J Cardiovasc Pharmacol* **14 (Suppl 11):**S13–S22.
Fonnum F (1975) A rapid radiochemical method for the determination of choline acetyltransferase. *J Neurochem* **24:**407–409.
Good PF, Hsu A, Werner P, Perl DP, and Olanow CW (1998) Protein nitration in Parkinson's disease. *J Neuropathol Exp Neurol* **57:**338–342.
Hersh LB, Wainer BH, and Andrews LP (1984) Multiple isoelectric and molecular weight variants of choline acetyltransferase. Artifact or real? *J Biol Chem* **259:**1253–1258.
Israël M, Manaranche R, Marsal J, Meunier FM, Morel N, Frachon P, and Lesbats B (1980) ATP-dependent calcium uptake by cholinergic synaptic vesicles isolated from *Torpedo* electric organ. *J Membr Biol* **54:**115–126.
Koppal T, Drake J, Yatin S, Jordan B, Varadarajan S, Bettenhausen L, and Butterfield DA (1999) Peroxynitrite-induced alterations in synaptosomal membrane proteins: insight into oxidative stress in Alzheimer's disease. *J Neurochem* **72:**310–317.
Kuhn DM and Geddes TJ (1999) Peroxynitrite inactivates tryptophan hydroxylase via sulfhydryl oxidation. Coincident nitration of enzyme tyrosyl residues has minimal impact on catalytic activity. *J Biol Chem* **274:**29726–29732.
Laage R and Langosch D (1997) Dimerization of the synaptic vesicle protein synaptobrevin (vesicle-associated membrane protein) II depends on specific residues within the transmembrane segment. *Eur J Biochem* **249:**540–546.
Lymar SV, Jiang Q, and Hurst JK (1996) Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry* **35:**7855–7861.
Morel N, Israël M, Manaranche R, and Mastour-Frachon P (1977) Isolation of pure cholinergic nerve endings from *Torpedo* electric organ. Evaluation of their metabolic properties. *J Cell Biol* **75:**43–55.

Morot Gaudry-Talarmain Y, Moulian N, Meunier FA, Blanchard B, Angaut-Petit D, Faille L, and Ducrocq C (1997) Nitric oxide and peroxynitrite affect differently acetylcholine release, choline acetyltransferase activity, synthesis, and compartmentation of newly formed acetylcholine in *Torpedo marmorata* synaptosomes. *Nitric Oxide* **1:**330–345.
Mothet JP, Fossier P, Tauc L, and Baux G (1996) Opposite actions of nitric oxide on cholinergic synapses: which pathways? *Proc Natl Acad Sci USA* **93:**8721–8726.
Muriel P and Sandoval G (2000) Nitric oxide and peroxynitrite anion modulate liver plasma membrane fluidity and Na(+)/K(+)-ATPase activity. *Nitric Oxide* **4:**333–342.
Nakane M, Schmidt HH, Pollock JS, Forstermann U, and Murad F (1993) Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett* **316:**175–180.
Oda Y (1999) Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. *Pathol Int* **49:**921–937.
Okuda T, Haga T, Kanai Y, Endou H, Ishihara T, and Katsura I (2000) Identification and characterization of the high-affinity choline transporter. *Nat Neurosci* **3:**120–125.
O'Regan S (1988) Binding of [3H]hemicholinium-3 to the high-affinity choline transporter in electric organ synaptosomal membranes. *J Neurochem* **51(6):**1682–1688.
Pryor WA and Squadrito GL (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am J Physiol* **268:**L699–L722.
Radi R, Peluffo G, Alvarez MN, Naviliat M, and Cayota A (2001) Unraveling peroxynitrite formation in biological systems. *Free Radic Biol Med* **30:**463–488.
Sbia M, Synguelakis M, Le Gal la Salle G, and Morel N (1991) Tubulin distribution along the *Torpedo* electroneuron. An immunochemical study. *Neurochem Int* **19:**355–362.
Smith MA, Richey Harris PL, Sayre LM, Beckman JS, and Perry G (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J Neurosci* **17:**2653–2657.
Souza JM, Giasson BI, Chen Q, Lee VM, and Ischiropoulos H (2000) Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J Biol Chem* **275:**18344–18349.
Tien M, Berlett BS, Levine RL, Chock PB, and Stadtman ER (1999) Peroxynitrite-mediated modification of proteins at physiological carbon dioxide concentration: pH dependence of carbonyl formation, tyrosine nitration, and methionine oxidation. *Proc Natl Acad Sci USA* **96:**7809–7814.
Trotti D, Rossi D, Gjesdal O, Levy LM, Racagni G, Danbolt NC, and Volterra A (1996) Peroxynitrite inhibits glutamate transporter subtypes. *J Biol Chem* **271:**5976–5979.
Uppu RM and Pryor WA (1996) Synthesis of peroxynitrite in a two-phase system using isoamyl nitrite and hydrogen peroxide. *Anal Biochem* **236:**242–249.
Viner RI, Williams TD, and Schoneich C (1999) Peroxynitrite modification of protein thiols: oxidation, nitrosylation, and S-glutathiolation of functionally important cysteine residue(s) in the sarcoplasmic reticulum Ca-ATPase. *Biochemistry* **38:**12408–12415.
Wu D and Hersh LB (1994) Choline acetyltransferase: celebrating its fiftieth year. *J Neurochem* **62:**1653–1663.

Address correspondence to: Dr. Yvette Morot-Gaudry-Talarmain, Laboratoire de Neurobiologie Cellulaire et Moléculaire, Center National de la Recherche Scientifique, 91198 Gif-sur-Yvette-Cedex, France. E-mail: morot@nbcm.cnrs-gif.fr.