Antagonism of botulinum toxin type A-induced cleavage of SNAP-25 in rat cerebral synaptosome by toosendanin

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Abstract Toosendanin (TSN), a triterpenoid derivative extracted from Chinese traditional medicine, has been demonstrated to be an effective cure for experimental botulism. This study is designed to explore its antibotulismic mechanism by Western blotting. The results showed that TSN incubation did not change the electrophoresis pattern and the amounts of synaptosomal-associated protein of 25 kDa (SNAP-25), syntaxin and synaptobrevin/vesicle-associated membrane protein in rat cerebral synaptosomes, but made the synaptosomes completely resistant to botulinum neurotoxin A (BoNT/A)-mediated cleavage of SNAP-25. After binding of BoNT/A to synaptosomes, TSN still partially antagonized the toxin-mediated cleavage of SNAP-25. However, TSN-incubated synaptosomal membrane fraction did not resist the cleavage of SNAP-25 by the light chain of BoNT/A. It is suggested that the antibotulismic effect of TSN results from blocking the toxin's approach to its enzymatic substrate.

Key words: Toosendanin; Botulinum toxin type A; Antibotulismic mechanism; Western blotting; Synaptosomal-associated protein of 25 kDa; Synaptosome

1. Introduction

The botulinum neurotoxins (BoNTs) are the most lethal biotoxins known to mankind and are responsible for the neuroparalytic disease botulism. Seven distinct serotypes of toxin have been identified and designated types A–G [1]. It is well established that the principal target of BoNT is the cholinergic nerve endings of neuromuscular junctions, where inhibition of acetylcholine release results in neuromuscular blockade and paralysis [2]. Each serotype of BoNT consists of a ~100 kDa heavy chain (HC) and a ~50 kDa light chain (LC), linked by a single disulfide bound and non-covalent forces [3,4]. A four-step mechanism consisting of binding, internalization, translocation and cleaving soluble NSF accessory protein receptor (SNARE) protein is currently the accepted view to explain BoNT intoxication [5]. The HC is responsible for the binding of the toxin to the nerve terminal and for internalization of the LC to the cytosol. The LC is a zinc-dependent endopeptidase and cleaves one of three SNARE proteins associated with transmitter release: synaptobrevin/vesicle-associated membrane protein (VAMP), synaptosomal-associated protein of 25 kDa (SNAP-25) and syntaxin. Syntaxin is the target for BoNT/C, VAMP for BoNT/B, /D, /F and /G, and SNAP-25 for BoNT/A, /C and /E [6,7].

Although cases of botulism have decreased considerably, sporadic outbreaks are still reported every year around the world. Moreover, due to their extreme toxicity and ease of production, the BoNTs are considered to be formidable threat agents. Currently, the approved treatment for botulinum intoxication is infusion of equine antitoxin. However, the antitoxin is only effective in the early stage of BoNT exposure, and no one can recognize all toxin serotypes and produce universal neutralization. Since the 1890s, numerous attempts have been made to develop approved pharmacological treatments for BoNT intoxication, but these efforts have met with little success. For example, K^+ channel blockers, lectins, H^+-permeant ionophores, zinc-dependent metalloprotease inhibitors and heavy metal chelators have been tested to evaluate their antibotulismic effects [8–15]. Some of these compounds were demonstrated to be effective in reversing the paralytic actions of BoNT temporarily, but most of them only delayed the time-to-block or had serious toxic side effects. Clearly, a safe and reliable drug, especially one that would be effective after BoNT poisoning, would be desirable.

Toosendanin ($C_{30}H_{38}O_{11}$, MW = 574, TSN) is a plant-derived triterpenoid derivative extracted from the bark of *Melia toosendan* Seib et Zucc [16,17]. It has been used in Chinese traditional medicine as an ascarifuge [18]. In the 1980s, both in vitro and in vivo experiments revealed that TSN is an effective antibotulismic agent. For example, TSN can for several hours prevent the death of animals (mice, rats and monkeys) that have been administered lethal doses of BoNT/A or /B [19]; the paralysis time of neuromuscular junction preparations induced by BoNT/A was lengthened several times after the preparations were incubated with TSN [20,21]. In particular, incubation with TSN gave the preparations a high tolerance to BoNT. Moreover, a high tolerance to BoNT can be observed in neuromuscular preparations isolated from rats which had received a single injection of TSN, and is associated with the TSN-induced increase in the frequency of miniature end-plate potential over time [22]. Further studies have shown that TSN inhibited various kinds of K^+ channels, such

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Abbreviations: BoNT, botulinum neurotoxin; HC, heavy chain; LC, light chain; TSN, toosendanin; HBM, HEPES-buffered medium; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble NSF accessory protein receptor; VAMP, vesicle-associated membrane protein

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as fast K^+ channels in mouse motor nerve terminals [23], delayed rectifier K^+ channels in neuroblastoma×glioma NG108-15 cells [24], inward rectifier K^+ channels and large- and small-conductance Ca^{2+}-activated K^+ channels in rat hippocampal neurons [25-27]. The inhibition of K^+ currents provides one of the explanations for TSN-induced initial facilitation of transmitter release and antibotulismic effect. The present study reports our recent finding that TSN treatment makes synaptosomes resistant to BoNT/A-mediated proteolytic cleavage on their SNAP-25 by the Western blot method.

2. Materials and methods

2.1. Evaluation of curative effect

To confirm the therapeutic effect of TSN against animal botulism, we duplicated the experiments reported by Li et al. [19]. Kunming mice (male, 18–20 g, supplied by Shanghai Experimental Animal Center, Chinese Academy of Sciences) were injected s.c. with BoNT/A (5 LD_{50}/mouse) and were randomly divided into a BoNT/A-injected group and a TSN treatment group with 10 mice per group. One hour later, each mouse in the TSN treatment and BoNT/A-injected groups was injected i.p. with TSN (8 mg/kg) or solvent (60% propane-1,2-diol) alone respectively. The survival time of each mouse was recorded and the survival rate in two groups was counted. All experiments conformed to the guidelines of the NIH on the ethical use of animals and all experimental procedures were reviewed and approved by the Animal Care and Use Committee of Shanghai Institutes for Biological Sciences. All efforts were made to minimize animal suffering.

2.2. Synaptosome preparation

Crude synaptosomes were prepared using the procedure described previously [28]. Briefly, male Sprague–Dawley rats (provided by Shanghai Experimental Animal Center, Chinese Academy of Sciences), weighing about 220 g, were killed by cervical dislocation and decapitation. The cerebral cortices were isolated and the myelin layer scraped off before homogenization in 0.32 M sucrose (320 mM sucrose, 5 mM HEPES-NaOH, pH 7.4). The homogenate was spun at 3000×*g* for 3 min in a Hitachi CR21G centrifuge and the supernatants were spun for 12 min at 14 600×*g*. The whiter loosely compacted layer of pellet was resuspended in 0.32 M sucrose and gently diluted 1:3 into a HEPES-buffered medium (HBM, in mM: NaCl 140, KCl 5, HEPES 20, $NaHCO_3$ 5, $MgCl_2$ 1, Na_2HPO_4 1.2, $CaCl_2$ 1.3 and glucose 10, pH 7.4). After centrifugation at 11 000×*g* for 10 s, the synaptosomes were pelleted. The pellet was resuspended in HBM to give a synaptosomal protein concentration of 1.5 mg/ml and stored at 0°C until use. The protein concentrations were determined by the Bradford method [29].

2.3. Synaptosomal membrane preparation

The synaptosomal membrane fraction was isolated as described previously [30]. Briefly, synaptosomes resuspended in HBM were diluted by adding 9 volumes of distilled water and then homogenized six times at 2000 rpm. The homogenate was centrifuged at 12 000×*g* for 12 min. The pellet, i.e. the synaptosomal membrane fraction, was resuspended in membrane buffer (in mM: HEPES-NaOH 20, NaCl 100, dithiothreitol 10 and 0.05% Triton X-100, pH 7.0) to a required concentration.

2.4. Sample treatment and Western blot analysis

In the experiments to observe if TSN affects the amounts of SNARE proteins in synaptosomes (Section 3.2), the isolated synaptosomes were incubated in HBM with (1×10^{-5} g/ml) or without TSN at 37°C for 2 h. In other experiments to determine if TSN affects cleavage of SNAP-25 by BoNT/A or its LC (Sections 3.3, 3.4 and 3.5), the samples, synaptosomes or synaptosomal membrane fraction, were incubated in various solutions and with different protocol (see Figs. 2A–4A). For control groups, equal solvents were added.

After incubation, the samples were lysed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer (5% SDS, 5% 2-mercaptoethanol, 50% glycerol, 0.02% bromophenol blue) and boiled for 5 min. After being vibrated and centrifuged at 12 000×*g* for 10 s, the lysates were resolved by 12.5% SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride (Pharmacia) membranes in Tris-glycine transfer buffer (25 mM Tris–HCl, 92 mM glycine, 20% methanol, 0.01% SDS). All subsequent experiments were done at room temperature (25°C). The membranes were blocked for 1 h with 5% bovine serum albumin in TBST (Tris 2.42 g/l, NaCl 8 g/l, Tween 20 1 ml/l). The membranes were washed three times with TBST (3×5 min) and incubated with primary antibody for 1 h. Following the primary antibody incubation, the membranes were washed with TBST (3×10 min) and then incubated in a horseradish peroxidase-conjugated secondary antibody for 1 h. This incubation step was terminated with three washes (3×10 min) and the immunoreactive protein bands were visualized using Western Blotting Luminol Reagent (Santa Cruz) according to the manufacturer's instructions. Membranes were exposed to film (Kodak X-Omat K Film) for periods adequate to visualize chemiluminescence bands.

2.5. Materials

TSN used in this work was a sample recrystallized in ethanol with a purity >98% [24]. Mouse monoclonal antibodies to SNAP-25, syntaxin and synaptobrevin/VAMP were purchased from Synaptic Systems Company (Germany) and used at dilution ranging between 1:20 000 and 1:200 000. Peroxidase-conjugated goat anti-mouse IgG (Calbiochem) was used at a dilution of 1:20 000. BoNT/A (~1.8×10^7 mouse LD_{50}/mg) was a gift from Professor Li P.Z., Chinese Academy of Military Medicine. BoNT/A was dissolved in phosphate-buffered saline (52.6 mM NaH_2PO_4, 26 mM Na_2HPO_4, 0.2% gelatin, pH 6.5) and aliquoted and stored at −20°C. For the synaptosomal membrane fraction, BoNT/A was treated in 10 mM dithiothreitol at 37°C for 30 min to separate the HC and LC [31,32]. All other reagents were of analytical grade.

2.6. Statistical analyses

Differences in protein immunoreactivity between two groups were determined by scanning densitometry (Scion Image, Scion Corporation, Frederick, MD, USA). The substrate cleavage data were expressed as the percentage difference in densitometry between two groups. All data are expressed as means ± S.E.M. Statistical analysis was performed using Student's *t*-test, and $P<0.05$ was considered significant.

3. Results and discussion

3.1. Saving botulismic mice from death

In 2–6 h after BoNT/A injection, botulism symptoms appeared in all of the animals. Then the mice in the control group successively died within 72 h. However, the symptoms of the animals in the experimental group progressively weakened, and finally disappeared. All mice in the TSN treatment group returned normal activity in 7 days.

The data obtained from the present and previous studies performed on intact animals [19] and isolated neuromuscular preparations [20,22] convince us that TSN is an effective cure of experimental botulism. TSN has been used as a clinical ascarifuge in China. It is possible to develop available drug to a cure for human botulism through studying TSN.

3.2. Having no influence on amounts of three SNARE proteins in synaptosomes

To observe if TSN affects amounts of SNARE proteins, isolated synaptosomes (0.75 mg protein/ml) were incubated with TSN (1×10^{-5} g/ml) or without TSN at 37°C for 2 h, then were analyzed by SDS–PAGE and immunoblotting (3.75 μg protein/lane, 12.5% gels) and the contents of syntaxin, SNAP-25 and VAMP were examined. It was found that there was no difference in electrophoresis pattern between the control and the TSN-incubated synaptosomes (Fig. 1A). In particular, in the TSN-treated synaptosomes, neither lost electrophoresis bands nor new ones could be observed. The amounts of syntaxin, SNAP-25 and VAMP in the TSN-incubated syn-

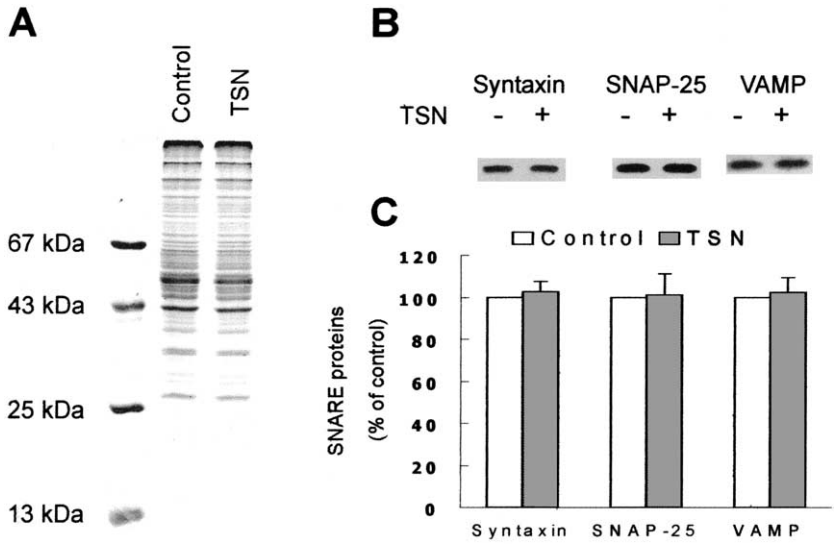

Fig. 1. TSN had no influence on the amounts of SNARE proteins in rat cerebral synaptosomes. Synaptosomes were incubated with (1×10^{-5} g/ml) or without TSN solution for 2 h at 37°C, and samples were analyzed by SDS–PAGE and immunoblotting (3.75 μg protein/lane, 12.5% gels). Neither the electrophoresis pattern (A) nor the amount of syntaxin, SNAP-25 or synaptobrevin/VAMP (B,C) of the TSN-treated group was different from that of the control ($P>0.05$). Data expressed in C are from 6–10 experiments.

aptosomes were 102.5 ± 4.9% ($n=8$), 102.2 ± 10.1% ($n=10$) and 101.2 ± 7.3% ($n=6$) of their controls, respectively (Fig. 1B,C). The results showed that TSN did not change the electrophoresis pattern of proteins and amounts of the three SNARE proteins in synaptosomes ($P>0.05$ vs. control), implying that TSN had no direct effects on SNARE proteins.

3.3. Eliminating proteolytic cleavage of SNAP-25 by BoNT/A in synaptosomes

As SNAP-25 is the proteolytic substrate of BoNT/A, a content decrease of SNAP-25 in synaptosomes incubated with BoNT/A can be detected by Western blotting. In our experimental condition, the amount of SNAP-25 decreased to 78.6 ± 6.1% of control after incubation with BoNT/A (0.4 mg/ml) ($n=6$, $P<0.001$, Fig. 2B,C). However, preincubation with TSN made the synaptosomes resistant to the proteolytic cleavage of SNAP-25 mediated by BoNT/A. The amount of SNAP-25 in the synaptosomes which were preincubated with TSN (1×10^{-5} g/ml) for 20 min before the incubation with BoNT/A was 98.0 ± 8.0% of the control ($n=6$, $P>0.05$ vs. control), indicating that TSN completely eliminated the action of BoNT/A to cleave its substrate. Decreasing the concentration of TSN to 5×10^{-7} g/ml, the antagonizing effect of TSN on the BoNT/A-induced cleavage of SNAP-25 was still observed (data not shown).

Previous studies performed on isolated neuromuscular junction preparations demonstrated that the antibotulismic site of TSN was at a neurotransmitter release process of synaptic transmission on which both TSN and BoNT, two presynaptic blockers, acted pharmacologically [20,22]. The present investigation further demonstrated that the antibotulismic effect of TSN resulted from its interfering with the cleavage of SNAP-25 by BoNT/A. After incubation with TSN, the synaptosomes resisted BoNT/A-mediated cleavage on their SNAP-25.

3.4. Having no direct effect on endopeptidase activity of BoNT/A light chain

To determine if the observation in Section 3.3, namely the

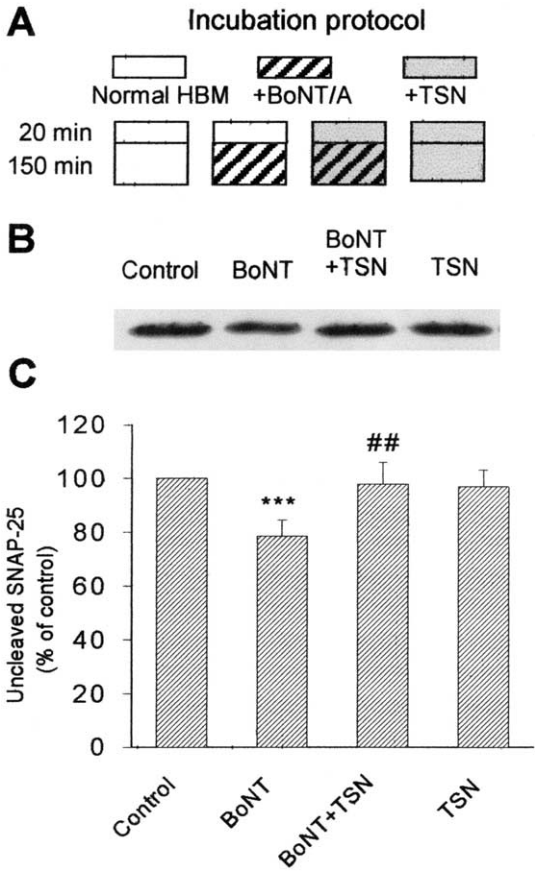

Fig. 2. TSN antagonized BoNT/A-induced cleavage of SNAP-25 in rat cerebral synaptosomes. After incubation in normal, BoNT/A (0.4 mg/ml)- or TSN (1×10^{-5} g/ml)-containing HBM (A), the synaptosomes were analyzed by SDS–PAGE and immunoblotting (3.75 μg protein/lane, 12.5% gels). TSN treatment made synaptosomes resistant to SNAP-25 cleavage induced by BoNT/A (B,C). Data expressed in C are from 6 experiments, $^{***}P<0.001$ vs. control, $^{\#\#}P<0.01$ vs. BoNT group, $P>0.05$ between BoNT/A+TSN group and control.

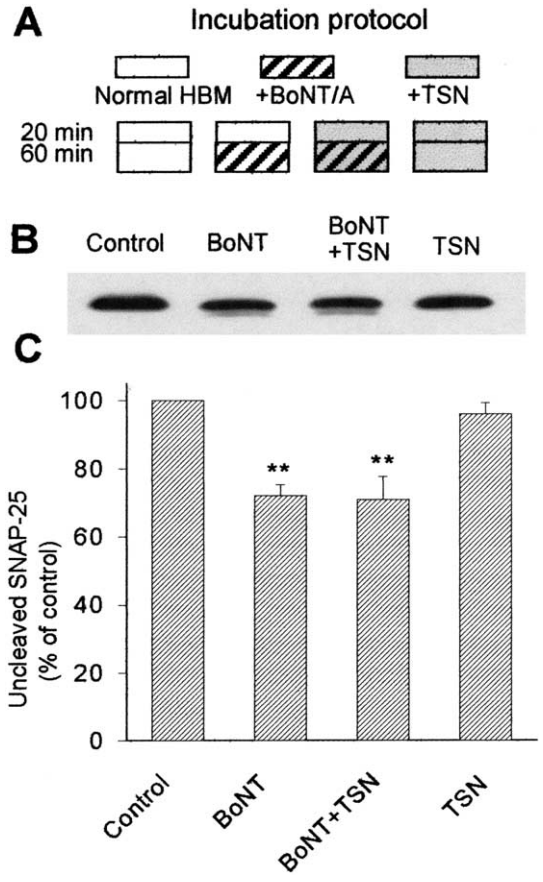

Fig. 3. TSN had no effect on dithiothreitol-treated BoNT/A-induced cleavage of SNAP-25 in rat cerebral synaptosomal membrane fractions. BoNT/A was reduced by 10 mM dithiothreitol at 37°C for 30 min to separate its heavy and light chains. After incubating the synaptosomal membrane fraction with reduced BoNT/A in different conditions (A), the samples were analyzed by SDS–PAGE and immunoblotting (3.75 μg protein/lane, 12.5% gels). TSN treatment did not affect cleavage of SNAP-25 in the membrane fraction by the reduced toxin (B,C). Data expressed in C are from four experiments. $^{**}P < 0.01$ vs. control, but $P > 0.05$ between BoNT and BoNT+TSN groups.

elimination of BoNT/A cleavage on SNAP-25 after TSN treatment, results from a direct inhibition of endopeptidase activity of LC of BoNT/A by TSN, similar experiments were performed but using synaptosomal membrane fractions instead of synaptosomes and reduced BoNT/A instead of intact BoNT/A (Fig. 3). In this case, in spite of preincubating the samples with or without TSN, SNAP-25 was cleaved similarly by the LC of BoNT/A. The amounts of remaining SNAP-25 were $72.2 \pm 3.2\%$ ($n = 4$) and $70.9 \pm 6.9\%$ ($n = 4$) of control respectively, indicating that TSN did not have a direct action on the endopeptidase activity of the LC of BoNT/A. The action of TSN is to interfere with the enzyme–substrate approach in a certain way.

3.5. *Partially antagonizing BoNT/A cleavage of SNAP-25 in synaptosomes after binding of the toxin*

The binding, as the first step of BoNT action, has been demonstrated to be temperature-independent and can be finished at 0°C [33]. Previous study showed that the binding of BoNT to synaptosomes could be equilibrated in 10 min at 0°C [34]. So, the step can be separated from subsequent temperature- and energy-dependent steps by controlling the incubation temperature.

In these experiments, synaptosomes were first incubated with BoNT/A (0.4 mg/ml)-containing solution at 0°C for 10 min, then at 37°C for 2 h after addition of TSN (1×10^{-5} g/ml) or corresponding buffer to the solution (Fig. 4A). The results showed that the amounts of SNAP-25 determined by Western blot in the BoNT/A intoxication group and TSN treatment group were $81.9 \pm 5.4\%$ and $92.0 \pm 8.7\%$ of control ($n = 10$) respectively. There was a significant difference between them ($P < 0.05$). The results indicate that TSN can still antagonize BoNT/A action to cleave SNAP-25 after binding of the toxin. Based on the fact that the amount of SNAP-25 in the TSN-treated group was significantly lower than that in the control group ($P < 0.05$), the antagonizing effect of TSN, after binding of BoNT/A, is only partial. The results also imply that an effect of TSN on binding of the toxin could not be excluded.

In conclusion, the obtained data show that the protective effect of TSN against BoNT/A-induced cleavage of SNAP-25 does not result from its direct inhibition of the endopeptidase activity of the toxin, but from its interference with the LC of the toxin–substrate approach in a certain way.

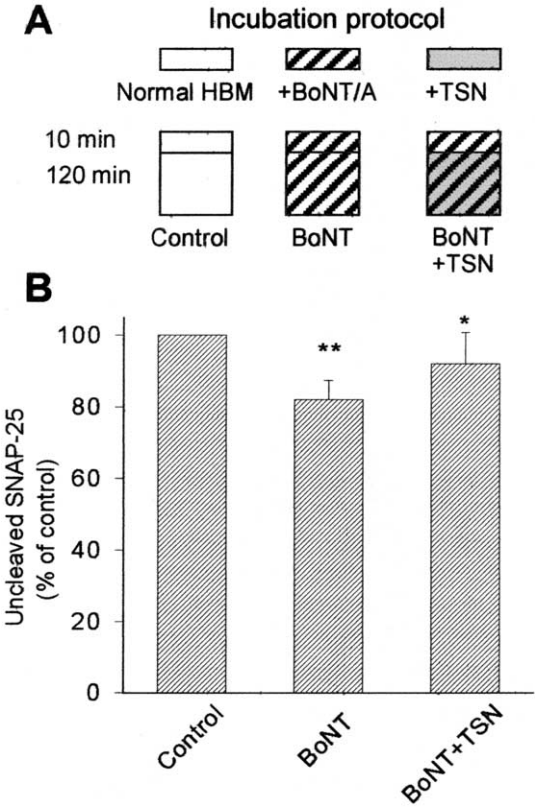

Fig. 4. TSN partially antagonized BoNT/A cleavage of SNAP-25 in rat cerebral synaptosomes after binding of the toxin. Synaptosomes were first incubated with BoNT/A at 0°C for 10 min, then incubated for 2 h at 37°C after adding TSN (A). The samples were analyzed by SDS–PAGE and immunoblotting (3.75 μg protein/lane, 12.5% gels). TSN made the synaptosomes partially resistant to the cleavage of SNAP-25 (B). Data expressed in B are from 10 experiments. $^{***}P < 0.001$ vs. control, $^{*}P < 0.05$ vs. BoNT group or vs. control.

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