Roles of Triton X-100 in NADPH-diaphorase Histochemistry

SHENGYUN FANG, JAMES CHRISTENSEN, JEFFREY L. CONKLIN, JOSEPH A. MURRAY, and GENE CLARK

Gastroenterology Research Laboratories, Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, Iowa.

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Introduction

The NADPH-diaphorase histochemical reaction involves the transfer of hydrogen from the substrate, NADPH, to a hydrogen acceptor, a tetrazolium salt, which is converted into the insoluble blue formazan (7,8). NADPH-diaphorase histochemistry stains a subpopulation of neurons in the central and enteric nervous systems (1,2,5,9,10,14,16–28). Evidence indicates that NADPH-diaphorase, so closely resembles nitric oxide synthase that they may be identical (6,27,28). Nitric oxide is now considered to be a neurotransmitter or neuromediator (4,12,13,23). NADPH-diaphorase histochemistry has become the most widely used method to study the nervous system (3,21).

Triton X-100 is widely but not universally used in NADPH-diaphorase histochemical staining. We investigated its effect on the staining and examined nitroblue formazan (NBF) production under the influence of Triton X-100. Exposure of opossum esophagus, intestine, and colon tissues to Triton X-100 before staining enhanced staining of nerve cells and fibers and suppressed staining of non-neural structures. Long exposures and high concentrations nearly abolished the staining of non-neural structures and decreased the staining of nerves. The use of an incubation medium containing Triton X-100 achieved the best staining of nerve cells and fibers. Addition of Triton X-100 to the incubation medium changed its color from yellow to purple; in the presence of tissues, this color change occurred much more quickly. Spectral analysis showed that Triton X-100 increases the rate of NBF formation in the presence of tissue supernatant. Triton X-100 increases it less in the absence of tissue supernatant. Therefore, Triton X-100 improves the histochemical staining, probably by catalyzing the activity of NADPH-diaphorase, by keeping the extracellular NBF in solution and thus suppressing the staining of non-neural structures, and by increasing the permeability of cell membranes. (J Histochem Cytochem 42:1519–1524, 1994)

Key Words: NADPH-diaphorase histochemistry; Triton X-100; Nitric oxide synthase; β-NADPH; NBT; Opossum; Esophagus; Intestine; Colon; Enzyme histochemistry.

Materials and Methods

Histochemistry

Tissue Preparation. Mature opossums of either sex were anesthetized with ketamine (60 mg/kg) followed by pentobarbital sodium (60 mg/kg).
Figure 1. Effects of Triton X-100 on NADPH-diaphorase histochemical staining. (A,C,E) Representative areas taken from three consecutive tangential sections (15 μm) stained under three different conditions. A and E were pre-treated with PB and C was pre-treated with 1% Triton X-100 for 30 min. Then the sections shown in A and C were stained using a normal incubation medium for 25 min. The section shown in E was stained with 1% Triton X-100 incorporated into the incubation medium for 25 min. The best staining of nerve fibers (arrows) was found in E, with improved staining in C and fewer stained nerve fibers (arrow) obscured by background staining in A. (B,D,F) Three ganglia stained under the three different conditions. D and F show the same ganglion in adjacent transverse sections (5 μm). The section in B was pre-treated with PB and those in D and F were pre-treated with 1% Triton X-100 for 30 hr. Then the sections in B and D were stained with normal incubation medium for 50 min. The section in F was stained with 1% Triton X-100 incorporated into the incubation medium for 50 min. The most strongly stained neurons (arrow) are seen in F. The weakest staining is in D. Both lightly stained neurons (arrowhead) and intensely stained neurons (arrows) were found in the section shown in B. Bars: A,C,E = 50 μm; B = 30 μm; D,F = 20 μm.
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The smooth-muscled part of the esophagus and parts of small intestine and colon were removed, washed with Krebs' solution equilibrated with 95% O₂-5% CO₂ at 37°C, patted flat to a sheet of rubber, and put into a freshly made solution of 4% paraformaldehyde in phosphate buffer (PB) at 4°C. Other parts of the smooth-muscled esophagus, small intestine, and colon were fixed in the form of a tube. After fixation for 1 hr, samples were washed with PB at 4°C for 1 hr (one change) and stored in 15% sucrose in PB at 4°C overnight. Flat samples were cut into 5-20-μm serial tangential sections and tubular preparations were cut into transverse sections on a cryostat.

Normal Incubation Medium for NADPH-diaphorase Histochromy. The normal incubation medium contained 1 mg/ml β-NADPH and 0.5 mg/ml nitroblue tetrazolium (NBT) (both from Sigma; St Louis, MO) in PB at pH 7.3.

Application of Triton X-100. Adjacent sections of the same thickness were used to investigate the effect of Triton X-100 on NADPH-diaphorase. A 20% stock solution of Triton X-100 (Fisher Scientific; Fair Lawn, NJ) in 0.1 M PB was used to make solutions of different concentrations of Triton X-100.

Pre-treatment. Solutions of 0.3, 0.5, 1, and 3% Triton X-100 were applied to cryostat sections at room temperature (RT) for 10 min to 1 hr before staining for NADPH-diaphorase. Pre-treated sections were then stained at RT for 10 min to 2 hr. As a control, 0.1 M PB was used as the pre-treatment solution instead of Triton X-100.

Use of a Triton X-100-containing Incubation Medium. An incubation medium itself containing 0.3, 0.5, 1, 2, and 3% Triton X-100 was used to react for NADPH-diaphorase at RT. Positive stained structures were found from 10 min to 2 hr. The control was NADPH-diaphorase histochemical staining without Triton X-100. The addition of Triton X-100 should be the last step when the incubation medium is made because Triton X-100 makes NBT hard to dissolve.

Post-treatment. After staining by the normal incubation medium (without Triton X-100), the sections were put into 3% Triton X-100 in 0.1 M PB (pH 7.3) overnight at 4°C.

Effect of Triton X-100 on Nerve Cell NADPH-Diaphorase Activity. Since the NADPH-diaphorase activity is very high, we pre-treated the sections with 0.1 M PB or 0.1 M PB containing 1% Triton X-100 for 30 hr at RT to decrease enzyme activity. The sections were then stained in normal incubation medium or incubation medium containing 1% Triton X-100.

Biochemical Assays

Spectral Analysis. Fresh smooth muscle layers of esophagus were dissected and then homogenized with a motor-driven Tenbroeck tissue grinder in ice-cold 0.1 M PB (pH 7.3) at 1 g/9 ml. The homogenate was centrifuged at 100,000 x g at 6°C for 60 min. The supernatant was used for analysis.

Spectral analysis for NBT reduction was done from 440 to 900 nm with the following solutions: (a) NBT/β-NADPH; (b) NBT/NADPH/Triton X-100; (c) nitroblue formazan (NBF) in 4% Triton X-100 diluted to 1% Triton X-100 (see below); (d) NBT/NADPH/supernatant; and (e) NBT/NADPH/Triton X-100/supernatant. All samples were in 0.1 M PB (pH 7.3). The final concentration of NBT was 0.5 mg/ml of NADPH was 1.0 mg/ml, and of Triton X-100 was 1% (v/v) in supernatant equivalent to 55.5 mg tissue (wet wt) per ml. pH was tested before and after the addition of Triton X-100. Absorbance of all the above solutions was read on a Beckman DU-20 spectrophotometer after 1-hr incubation at RT.

Since commercial NBF (Sigma n-6276) is difficult to dissolve in an aqueous solution, 0.1 ml of a saturated solution in dichloromethane was evaporated at RT under a stream of N₂ in a rotating test tube. To the resulting thin film of NBF we added 1.0 ml of Triton X-100 (4%). This was vortexed and allowed to stand overnight. After centrifuging at 1000 x g to remove undissolved NBF, an aliquot was diluted to contain 1% Triton X-100, which was then spectrally analyzed.

Colorimetric Analysis of NBF. As previously reported, the enzymatically reduced NBT formazan can be dissolved in the presence of Triton X-100 to form a colored solution and the color development occurs in a time- and concentration-dependent fashion (11,15). Therefore, the increases in absorbance at 535 nm were monitored in three different incubates: (a) NBT/β-NADPH/Triton X-100; (b) NBT/NADPH/Triton X-100/supernatant; and (c) NBT/NADPH/supernatant. The concentrations of NBT, NADPH, and Triton X-100 were the same as above. The amount of supernatant was 50% of the above amount. The incubation was done at RT. Before the absorbance was read, Triton X-100 was added to 1 ml of samples taken from Incubate c and the same amount of PB was added to Incubates a and b to ensure the same dilution and Triton X-100 concentration in the three incubates.

Results

Staining Without Triton X-100

The NADPH-diaphorase histochemical reaction without Triton X-100 stained most structures in the esophagus, small intestine, and colon. Two populations of stained neurons, intensely stained and lightly stained cells, were found in the myenteric (Figure 1B) and submucosal plexuses. Nerve fibers stained poorly in the submucosal plexuses. Nerve fibers stained poorly to moderately well (Figure 1A). Vascular smooth muscle cells, esophageal and intestinal epithelium, esophageal and intestinal gland cells, and interstitial cells of Cajal in esophagus were moderately well stained. Fibroblasts, mast cells, and smooth muscle cells were faintly stained. Extension of the incubation time increased staining of all stained structures, which obscured nerve fibers.

Staining after Pre-treatment with Triton X-100

With the same incubation time, pre-treatment of the sections with Triton X-100 enhanced staining of neurons and nerve fibers (Figure 1C), but there was no improvement in the staining of neurons in thin sections (Figure 1D). Staining of non-neural structures and most lightly stained neurons was greatly diminished or abolished (Figure 1C). Longer pre-treatment times and higher concentrations of Triton X-100 nearly abolished staining of non-neral structures and lightly stained nerve cells and decreased the number of stained nerve fibers and the staining of intensely stained nerve cells (Figure 1D). Sections pre-treated with 0.5–1% Triton X-100 for 30–60 min at RT showed much improved staining of nerve fibers. The time for an optimal stain, 30–90 min, varied with the thickness of sections. After completion of the stain, the incubation medium had not changed in color.

Staining with Triton X-100 in the Incubation Medium

After the addition of Triton X-100 to the normal incubation medium, its color changed from yellow to blue/purple in 10–30 min, varying with the concentration of Triton X-100 used, and the color then gradually intensified. In the presence of tissue, the color change took place much more quickly. When we separately put
0.3–3% Triton X-100 in a 0.5 mg/ml solution of NBT and in a 1 mg/ml solution of NADPH, the color of the solutions did not change.

With the NADPH-diaphorase incubation medium containing 0.5–3% Triton X-100, the staining of nerves was greatly enhanced and was better than that in pre-treated sections (Figures 1E and 1F), but the staining of non-neural structures varied with the different concentrations of Triton X-100. The use of higher concentrations (1–3%) of Triton X-100 resulted in weaker or no staining of non-neural structures, with excellent staining of nerves.

Staining After Post-treatment with Triton X-100
Sections were stained first for NADPH-diaphorase using the normal incubation medium and then they were put in 3% Triton X-100 in PB overnight. The staining of neurons, nerve fibers, and interstitial cells of Cajal of the circular muscle layer was improved, with decreased staining of the smooth muscle.

Spectral analysis of different reaction solutions revealed the same maximal absorbance at a wavelength of 535 nm as commercial NBF in 1% Triton X-100 solution (Figure 2), which indicates that the NBF was generated in all reaction mixtures except for NBT/NADPH solution without Triton X-100. One percent Triton X-100 in the reaction mixture was able to dissolve the enzymatically produced formazan. After incubation for 1 hr, the mixture NBT/NADPH/supernatant/Triton X-100 produced a dark-blue NBF solution. The mixture NBT/NADPH/supernatant formed a fine dark precipitate without a color change in the solution. After this mixture was centrifuged, the supernatant was discarded and re-suspended with 1% Triton X-100. The complete dissolution took about 10 min and finally formed a dark-blue solution. These two supernatant-containing mixtures generated about the same amount of NBF, which implies saturation of the reduction of NBT after 1-hr incubation in the two solutions.

Since spectral analysis of the different solutions including commercial NBF but not the solution with only NBT/NADPH in phosphate buffer showed similar absorbance profiles and similar absorbance maxima at about 535 nm (Figure 2), this supported the assumption that the color change reflected NBF formation. We investigated the reduction of NBT with time. We used half the previous amount of supernatant used above without a change in NBT, NADPH, and Triton X-100 concentrations. We examined the formation of NBF during 1 hr (Figure 3). From Figure 3 it can be seen that Triton X-100 non-enzymatically catalyzes the reduction of NBT by NADPH. The rate of NBF formation is greatest with both tissue supernatant and Triton X-100. The rate of NBT reduction by supernatant with Triton X-100 is much greater than the sum of the rates of reduction catalyzed by supernatant alone and by Triton X-100 alone, respectively. After 30 min of incubation, NBF formed by the supernatant with Triton X-100 was 150% of that formed by the supernatant without Triton X-100. After 1 hr it was still greater, with 115% of the NBF formed by supernatant without Triton X-100. The pH of all mixtures used did not change after the addition of Triton X-100.

Discussion
The use of Triton X-100 in NADPH-diaphorase histochemistry has been based on its function as a detergent. This study assessed its roles in NADPH-diaphorase histochemistry by observing its effect on staining and by using spectral analysis to estimate the amount of enzymatically produced formazan under the effects of Triton X-100.

The color changes in the solutions NADPH/NBT/Triton X-100 and NADPH/NBT/Triton X-100/sections (staining) were due to the dissolution of formazan in Triton X-100, confirmed by spectral analysis. The absorbance maxima in the solutions NADPH/NBT/Triton X-100 and NADPH/NBT/Triton X-100/crude enzymes (tissue homogenate) were the same as with commercially obtained for-
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in nerves? Probably the formation of NBF is much greater than the rate of dissolution. It cannot be excluded that Triton X-100 also inhibits some enzyme in the non-neural structures that catalyzes the reduction of NBT by NADPH. Long pre-treatment with Triton X-100 resulted in a decrease in the staining of neurons, which was probably attributed to the loss of enzyme from the sections.

Therefore, Triton X-100 non-enzymatically catalyzes the reduction of NBT by NADPH to form formazan. Triton X-100 is also an activator of NADPH-diaphorase. Triton X-100 decreases the binding of formazan to cell membranes and dissolves the extracellular formazan. Triton X-100 also increases the permeability of cell membranes. These effects together can account for the improvement in the histochemical staining produced by Triton X-100.

Literature Cited