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Exocytosis of Endothelial Cells Is Regulated by *N*-Ethylmaleimide-Sensitive Factor

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Summary

Endothelial exocytosis of granules is a rapid response to vascular injury. However, the molecular machinery that regulates exocytosis in endothelial cells is not well understood. Recently developed techniques have defined the endothelial proteins that control vesicle and granule trafficking in endothelial cells. These techniques have revealed that syntaxin 4, synaptobrevin 3, and *N*-ethylmaleimide-sensitive factor (NSF) play a critical role in endothelial granule exocytosis. Additional studies have shown that nitric oxide regulates exocytosis by chemically modifying NSF. Further characterization of the factors that regulate exocytosis will lead to novel treatments for vascular diseases such as myocardial infarction and stroke.

Keywords

N-Ethylmaleimide-sensitive factor; granule; nitric oxide; P-selectin; vesicle; von Willebrand factor; Weibel–Palade body

1 Introduction

The initial endothelial response to injury is exocytosis. A variety of stimuli, including hypoxia, physical trauma, or inflammatory mediators, trigger endothelial cells to release the contents of specialized granules called Weibel–Palade bodies (1). Von Willebrand factor (VWF), released by endothelial exocytosis, induces platelet rolling and thrombosis. P-Selectin externalized by exocytosis activates leukocyte rolling, the first step in leukocyte trafficking. Endothelial granules also contain additional proinflammatory and prothrombotic mediators that activate inflammation and thrombosis in response to vascular injury (2,3).

The exocytic machinery that drives vesicle trafficking and membrane fusion in endothelial cells is similar to that found in neurons and yeast (4). The adenosine 5'-triphosphate (ATP)-hydrolyzing enzyme *N*-ethylmaleimide-sensitive factor (NSF) interacts with transmembrane proteins called soluble NSF receptor attachment protein receptors (SNAREs) to regulate exocytosis. Unique SNARE molecules on the cytoplasmic surface of endothelial granules and on the cytoplasmic surface of the plasma membrane interact, specifying which particular granules fuse with a specific target membrane. Members of the superfamily of rab proteins guide the assembly of SNAREs and NSF onto the surface of granules in preparation for membrane fusion. A subset of SNAREs and rabs along with NSF regulate exocytosis in endothelial cells (5).

Exocytosis of granules from endothelial cells occurs within minutes of stimulation. Special techniques have been adapted to study the intracellular machinery that drives exocytosis and

the extracellular and in vivo consequences of exocytosis. The discovery of the molecular machinery that regulates exocytosis from endothelial cells has led to the invention of novel peptides that inhibit exocytosis by targeting these components (6,7). Using these tools, studies have characterized novel regulators of endothelial exocytosis (5,8,9). We found that nitric oxide (NO), an endogenous messenger molecule that inhibits inflammation, chemically modifies NSF. NO S-nitrosylation of NSF may explain how NO inhibits vascular inflammation. These techniques that characterize exocytosis have led to better understanding of the importance of endothelial exocytosis in the host response to vascular injury.

2 Materials

2.1 Endothelial Cell Culture

1. Endothelial cells analyzed for exocytosis include human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) from Cambrex (East Rutherford, NJ).
2. Endothelial media: Endothelial growth media 2 (EGM-2). EGM-2 is supplemented with a Bullet kit supplement (Cambrex) containing growth factors and cytokines and supplemented with 2% fetal bovine serum (FBS; Cambrex).
3. Endothelial cells are grown on sterile 100-mm tissue culture dishes (Corning Life Science, Acton, MA) without matrix.
4. Solutions of trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA) (1 mM) and phosphate-buffered saline (PBS), pH 7.4, used were from Gibco/Invitrogen (Carlsbad, CA).
5. Thrombin stock: 100 U/mL containing 1% bovine serum albumin (BSA); store at -80°C . Thrombin stocks are defrosted once, and then the excess is discarded, never refrozen or rethawed.
6. Diethylenetriamine (DETA)-NONOate (Cayman Chemicals, Ann Arbor, MI): DETA-NONOate is dissolved in 10 mM NaOH solution just before adding to the media. DETA-NONOate is very sensitive to the temperature and pH of the solution. It also has a half-life of 20 h at 37°C . DETA-NONOate powder should be dissolved in buffer on ice just before use (*see* Note 1).

2.2 Enzyme-Linked Immunosorbent Assay and Fluorescence Activated Cell Sorting Materials

1. Enzyme-linked immunosorbent assay (ELISA) kits to measure exocytosis of VWF from endothelial cells (American Diagnostica, Stamford, CT).
2. Antibody to P-selectin was a mouse monoclonal antibody immunoglobulin G1 (IgG1) isotype (BD Biosciences Pharmingen, San Jose, CA, 555524). Its isotype-matched control is also from BD Biosciences Pharmingen (555749).

2.3 Immunoprecipitation for S-Nitrosylated NSF

1. Cell lysis buffer: 50 mM Tris-HCl, 150 mM NaCl, 1% NP40, phenylmethylsulfonyl fluoride (PMSF), and protein inhibitor cocktail.

¹All NO donors are unstable. DETA-NONOate is very sensitive to the temperature and pH of the solution. DETA-NONOate has a half-life of 20 h at 37°C . DETA-NONOate powder should be dissolved on ice just before use. NO donors like DETA-NONOate should never be heated since they might explode.

2. Control rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody to S-nitrosocysteine (Sigma, St. Louis, MO).
3. Protein A/G agarose (Santa Cruz Biotechnology).
4. Loading buffer (10X): 250 mM Tris-HCl, 1920 mM glycine, 1.0% (w/v) sodium dodecyl sulfate (SDS), pH 8.3; store at room temperature.
5. Transfer buffer (1X): 48 mM Tris-HCl, 39 mM glycine, 20% (v/v) methanol, 0.05% (w/v) SDS; store at room temperature.
6. Tris-buffered saline with Tween (TBS-T): Prepare 10X stock with 1.37 M NaCl, 27 mM KCl, 250 mM Tris-HCl, pH 7.4, 0.5% Tween-20. Dilute 100 mL with 900 mL water for use.
7. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
8. Primary antibody dilution buffer: TBS-T supplemented with 1% (w/v) nonfat dry milk.
9. Antibody to NSF (BD Transduction Laboratories, San Jose, CA).
10. Secondary antibody dilution buffer: TBS-T supplemented with 2.5% (w/v) nonfat dry milk.
11. Secondary antibody is anti-mouse IgG conjugated to horse radish peroxidase (HRP) (Santa Cruz Biotechnology).

2.4 S-Nitrosylation of NSF by Biotin-Switch

1. Cell lysis buffer: 250 mM HEPES-NaOH, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine.
2. Control rabbit IgG (Santa Cruz Biotechnology). Antibody to S-nitrosocysteine (Sigma).
3. Protein A/G agarose (Santa Cruz Biotechnology).
4. Loading buffer (10X): 250 mM Tris-HCl, 1920 mM glycine, 1.0% (w/v) SDS, pH 8.3; the buffer is stored at room temperature.
5. Transfer buffer (1X): 48 mM Tris-HCl, 39 mM glycine, 20% (v/v) methanol, 0.05% (w/v) SDS; store at room temperature.
6. Tris-buffered saline with Tween (TBS-T, 10X): 1.37 M NaCl, 27 mM KCl, 250 mM Tris-HCl, pH 7.4, 0.5% Tween-20. Dilute 100 mL with 900 mL water for use.
7. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
8. Primary antibody dilution buffer: TBS-T supplemented with 1% (w/v) nonfat dry milk.
9. Antibody to NSF (BD Transduction Laboratories).
10. Secondary antibody dilution buffer: TBS-T supplemented with 2.5% (w/v) nonfat dry milk.
11. Secondary antibody is antimouse IgG conjugated to HRP (Santa Cruz Biotechnology).

2.5 Leukocyte Adhesion

1. HL-60 cells (American Type Culture Collection, Manassas, VA).

2. 3'-O-Acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester (BCECF-AM) (Molecular Probes/Invitrogen, Carlsbad, CA).
3. Anti-P-selectin antibodies (purchased from BD Bioscience Pharmingen).

2.6 TAT Fusion Peptides

1. TAT fusion peptides are composed of three parts (10). The N-terminal part is an 11-amino acid fragment from the human immunodeficiency virus (HIV) TAT peptide. The middle portion contains three glycine residues as a linker to decrease steric hindrances. The C-terminal portion consists of 20–30 amino acids that correspond to rationally selected domains of proteins that regulate exocytosis.

2.7 Leukocyte Rolling

1. Solutions of ketamine (100 mg/mL) and xylazine (100 mg/mL) are prepared in PBS.
2. Rhodamine 6 G 0.05% is prepared in PBS.

3 Methods

3.1 Endothelial Cell Culture

1. Culture HUVECs or HAECs in EGM-2 medium supplemented with the Bullet kit growth factors and serum.
2. Change the growth medium the day after seeding and every other day thereafter (*see* Note 2).

3.2 ELISA for VWF Release

1. Plate HUVECs or HAECs from passages 2–4 into two 24-well plates with 250 μ L medium per well or into three 96-well plates with 100 μ L medium per well. Feed cells with endothelial medium supplemented with EGM-2 and serum and Bullet kit. Grow overnight.
2. Make sure cells are confluent the next morning. Remove the tissue culture plates from the incubator and place in a tissue culture hood on top of Styrofoam slabs to maintain the temperature at 37 °C. Do not shake cells or move plates quickly because sudden movements will cause exocytosis. Change the medium with prewarmed EGM-2 medium without serum and without Bullet kit supplements (*see* Note 3).
3. Add 1.0 U/mL thrombin and move plates back into the tissue culture incubator. Incubate for 30–60 min.
4. Carefully remove plates from the incubator, moving plates gently as above and placing plates on Styrofoam slabs in a tissue culture hood to maintain an even temperature.
5. Harvest supernatant. Freeze supernatant.

²HAECs and HUVECs grow slowly, and HAECs will not proliferate past passage numbers 7–8. We usually use endothelial cells at less than passage 5.

³Endothelial cells should be handled very carefully since we have found that a variety of physical stimuli can activate exocytosis. For example, the metal base of tissue culture hoods is cooler than 37 °C and conducts heat away from endothelial cells and their media; this cooling activates exocytosis. Therefore, we place our cell cultures on top of Styrofoam slabs in the tissue culture hood and leave the cells exposed to room temperature for as short a time as possible. Shear stress can activate exocytosis, so we add media to the side of the endothelial tissue culture dish instead of pipeting media directly on top of endothelial cells. Abrupt motion (such as rapping the side of the tissue culture dish or dropping the dish onto the tissue culture incubator base) can also activate exocytosis. Thus, endothelial cells need to be treated gently during studies of exocytosis.

6. Add supernatant to VWF ELISA and add cell media standards. Watch the assay carefully; the moment the color of any sample turns blue, stop the entire assay with stop buffer.
7. Measure the OD at 450 nm in a spectrophotometer. (An example of using the ELISA to measure VWF release is shown in Fig. 1.)

3.3 FACS for P-Selectin Externalization

1. Culture HUVECs or HAECs in EGM-2 medium with Bullet kit growth factors and serum. Use passages 2–5 endothelial cells. Higher-passage cells lose the ability to undergo regulated exocytosis.
2. Plate cells on a 12- or 24-well plate.
3. Culture cells for 1–2 d until confluent.
4. Remove endothelial cells from incubator. Handle the cells carefully as above, without sudden motions, and place on Styrofoam slabs inside a tissue culture hood to maintain the temperature.
5. Refeed the cells with EGM-2 medium without growth factors or serum. Add medium gently to cells by pipeting on the side of the dish rather than the base so that the jet of medium does not stimulate the endothelial cells.
6. Add 0.5 U/mL thrombin.
7. Gently replace cells in the incubator for 30 min (*see* Note 4).
8. Gently remove cells from the incubator and place on Styrofoam slabs in a tissue culture hood.
9. Aspirate medium and wash cells with 1 mL prewarmed PBS twice.
10. Add antibody to P-selectin or an isotype-matched control antibody at 10 μ L antibody per 1 mL PBS cell medium.
11. Incubate in the dark at 22 °C for 1 h.
12. Wash cells three times with 1 mL PBS. Trypsinize cells for 3 min. Add EGM-2 with fetal calf serum to inactivate trypsin. Transfer cells to Eppendorf 1.5-mL tubes.
13. Centrifuge and resuspend in PBS three times.
14. Centrifuge cells, resuspend in 500 μ L PBS, and immediately analyze by FACS.

3.4 Measurement of NSF S-Nitrosylation by Immunoprecipitation

1. Grow endothelial cells as above.
2. Treat cells with 0, 0.1, 0.5, and 1 mM DETA-NONOate and culture for 16 h without light (*see* Note 5).
3. After 16 h, aspirate cell medium and rinse cells with cold PBS.
4. Add 0.7 mL lysis buffer to the cells in the 100-mm dish. Rock the dish gently for 15 min at 4 °C. Scrape cells and transfer the resulting lysate to an Eppendorf tube.

⁴P-Selectin is a challenging marker to study for exocytosis since after externalization it is rapidly internalized. We treat endothelial cells with thrombin for 10–30 min but no longer than 30 min when analyzing P-selectin externalization.

⁵S-Nitrosocysteine bonds are unstable; light cleaves the cysteine–NO bond, so the immunoprecipitation should be performed in the dark.

5. Wash the dish once with 0.3 mL lysis buffer and combine with first lysate. Incubate 15 min on ice.
6. Centrifuge cell lysate at 10,000 *g* for 10 min at 4 °C. Transfer the supernatant to a new Eppendorf tube and put on ice.
7. Measure the protein concentration of each sample. Prepare the diluted BSA standards in the range of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL. Pipet 10 µL of these standards and each HAEC lysate into appropriately labeled 1.5-mL Eppendorf tubes. Add 300 µL of the Coomassie reagent to each tube and vortex. Wait for a few minutes until you can see that the color developed well and read at 595 nm with the spectrophotometer. Prepare a standard curve by plotting each BSA datum. Using this curve, determine the protein concentration of each HAEC lysate. The reagents should be warmed to room temperature before use as cold temperatures may affect the assay.
8. To preclear cell lysates, add 0.25 µg of the control rabbit IgG and 20 µL of suspended protein A/G-agarose to the whole cell lysate. Incubate at 4 °C for 60 min.
9. After centrifuging lysates at 1000 *g* for 30 s at 4 °C, transfer supernatant (cell lysate) to a new Eppendorf tube on ice.
10. Add 10 µL anti-S-nitrosocysteine antibody and rotate the lysates slowly for 2–24 h at 4 °C in the dark (*see* Note 5).
11. Add 20 µL suspended protein A/G-agarose and incubate for 1 h at 4 °C on a rotator.
12. Spin 1 min at 1000 *g* at 4 °C and aspirate and discard supernatant.
13. Add 500 µL lysis buffer and invert the Eppendorf tube several times.
14. Spin 1 min at 1000 *g* at 4 °C, aspirate and discard supernatant, and resuspend in lysis buffer.
15. Repeat wash steps three times.
16. After final wash, resuspend beads in 50 µL Laemmli sample buffer and vortex.
17. Boil samples for 2–3 min.
18. Prepare the loading buffer by diluting 50 mL 10X running buffer with 450 mL water in a measuring cylinder. Cover with Parafilm and invert to mix.
19. Prepare a 1.5-mm thick 7.5% gel by cutting the seal of the bottom edge. Set the gel and remove the comb carefully.
20. Add the running buffer to the upper and lower chambers of the gel unit and load the 40 µL of each sample in a well. One well is used for the prestained molecular weight markers.
21. Complete the assembly of the gel unit and connect to a power supply.
22. Run at 30 mA until the blue dye fronts can be just run off the gel.
23. Transfer the samples (which have been separated by SDS polyacrylamide gel electrophoresis [PAGE]) to nitrocellulose membranes by the trans-blot machine.
24. Lay two more wetted sheets of watman chromatography grade 3 MM paper on top of the membrane, ensuring that no bubbles are trapped in the resulting sandwich. Close the transfer cassette.
25. Rinse the separating gel with PBS and place on top of the Whatman chromatography grade 3 MM paper.

26. Lay a sheet of the nitrocellulose cut just larger than the size of the separating gel on the surface of the gel.
27. Lay two more wetted sheets of Whatman chromatography grade 3 MM paper on top of the membrane, ensuring that no bubbles are trapped in the resulting sandwich. Close the transfer cassette.
28. Transfer proteins at 15 V for 30 min.
29. The colored molecular weight marker should be clearly visible on the membrane. The membrane can be cut to a good size using a razor blade.
30. Incubate the nitrocellulose in 40 mL blocking buffer for 1 h at room temperature on a rocking platform.
31. After the blocking buffer is discarded, add a 1:1000 dilution of the antibody to NSF in TBS-T/1% milk for 1 h at room temperature or overnight at 4 °C on a rocking platform.
32. Wash the membrane three times for 5 min each with 50 mL TBS-T.
33. Freshly prepare the HRP-conjugated secondary antibody for each experiment as a 1:2,000 dilution in TBS-T/2.5% milk and add it to the membrane for 60 min at room temperature on a rocking platform.
34. Wash the membrane three times for 15 min each with TBS-T.
35. Warm the enhanced chemiluminescence (ECL) reagent separately to room temperature. Once the final wash is removed from the blot, mix the ECL detection solutions A and B together in a ratio of 40:1 and then immediately add to the blot.
36. Wait for 1 min, wrap the membrane in the Saran wrap, and put it on an X-ray film cassette.
37. In the darkroom, place film on the membrane in an X-ray film cassette for a suitable exposure time, usually a few minutes. You can see the band around 80 kDa. (An example of immunoprecipitation to detect S-nitrosylation of NSF is shown in Fig. 2.) (See Note 6.)

3.5 Measurement of NSF S-Nitrosylation by Biotin-Switch Assay

1. Add 16 mL ice-cold assay buffer (250 mM HEPES-NaOH, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) to every gram of minced tissue (brain, heart, and spleen) and homogenize the tissue with a Polytron for about 30–45 s on ice.
2. Centrifuge the tube at 750 g for 10 min at 4 °C.
3. Add 40 µL 10% 3-([3-cholamidopropyl]dimethylamino)-1-propanesulfonate (CHAPS) per milliliter of supernatant to the mixture.
4. Dispense tissue homogenate into two 100-µL aliquots in 1.5-mL centrifuge tubes. As a positive control, add 10 µL of DEA-NONOate (10 mM) to the tissue homogenate. Mix the reaction mixture and incubate it in the dark for 30 min at room temperature.
5. Add the 300 µL blocking solution (9 volumes assay buffer, 1 volume 25% SDS solution, 20 mM methylmethanethiosulfonate) to the mixture. Suspend the mixture well and incubate for 1 h at 50 °C in the dark.

⁶Several techniques have been used to study NO modification of target proteins. This method uses immunoprecipitation of nitrosothiol groups followed by immunoblotting of the specific protein (in this case, NSF) (8). This technique is more rapid than other techniques but depends on the quality of the antibody to nitrosothiol.

6. Add cooled acetone (800 μ L at -20°C), incubate at -20°C for 10 min, then centrifuge at 2000 g for 10 min at 4°C .
7. After removal of acetone, rinse pellet with another 800 μ L cooled acetone and centrifuge again as described above.
8. Resuspend the pellets with 60 μ L solubilization buffer (assay buffer with 1% SDS) per tube.
9. Add 2 μ L freshly prepared ascorbate solution (50 mM) to the mixture and vortex vigorously.
10. Add 20 μ L 4 mM HPDP-biotin to the mixture. Vortex and incubate at room temperature for 1 h.
11. Add cooled acetone (100 μ L at -20°C), incubate at -20°C for 10 min, and then centrifuge at 2000 g for 10 min at 4°C .
12. Suspend pellet in 100 μ L solubilization buffer.
13. Add neutralization buffer (20 mM Tris-HCl pH 7.7, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) (200 μ L) and 15 μ L of streptavidin-agarose (neutravidin-agarose, Pierce). Rotate the mixture for 1 h at room temperature.
14. Centrifuge the mixture at 200 g at 10 s.
15. Wash the pellet with 100 μ L neutralization buffer containing 600 mM NaCl five times.
16. Add 20 μ L 4X SDS sample buffer to the pellet and load onto a 7.5% acrylamide gel for SDS-PAGE.
17. Blot the gel onto nitrocellulose membrane and perform Western blotting analysis with a monoclonal antibody to NSF. (An example of the biotin-switch assay used to detect S-nitrosylation of NSF is shown in Fig. 3.) (See Note 7.)

3.6 Fluorescent Microscopy for Leukocyte Adhesion

1. Grow HL-60 cells in RPMI 1640 medium with 10% FBS. Spin cells down and resuspend in HBSS solution.
2. Culture HUVECs (2×10^5) on 12-well plates at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Cells should be 100% confluent before the experiment begins.
3. One of the endothelial cell wells should be pretreated with 1 μ g of antibody to P-selectin for 15 min in the incubator. This serves as a negative control.
4. After 10 min, stimulate HUVECs with 0.5 U/mL thrombin for 45 min.
5. Calculate the HL-60 cell number using the hemocytometer cell count calculator. Adjust the HL-60 cell number to $1 \times 10^7/\text{mL}$.
6. Label HL-60 cells with 10 μ M BCECF-AM for 15 min at 37°C in the incubator.
7. Wash HL-60 cells with 5 mL warm HBSS solution twice.
8. Adjust the final HL-60 cell number to $1 \times 10^8/\text{mL}$ and keep them in the incubator until use.

⁷This method of studying NO modification of target proteins relies on blocking all free-thiol groups, followed by removal of NO from nitrosothiols; the free thiols are then reacted with a compound containing biotin so that the target proteins can be precipitated. This technique, often called the Jaffrey technique or the biotin-switch assay, is more lengthy than other techniques but is more specific (11).

9. Add 0.5 mL HL-60 cells to HUVECs after HUVECs have been stimulated with thrombin for 45 min as above.
10. Cover the plate with aluminum foil to prevent photobleaching and leave it at 4 °C for 15 min to allow for cell adhesion.
11. Wash the HL-60/HUVEC mixture three times with fresh HBSS solution very gently to remove unbound cells.
12. Add EGM-2 medium.
13. Count the HL-60 cells adhering to endothelial cells in three to five randomly selected fields of view in each well by a fluorescent microscope.
14. Photograph the fluorescence intensity with a fluorescent microscope at $\times 10$ over an area $520 \times 700 \mu\text{m}$. (An example of fluorescent microscopy to detect leukocyte adhesion to endothelial cells is shown in Fig. 4.)

3.7 TAT Fusion Peptides to Inhibit Exocytosis

1. Dissolve TAT fusion polypeptides in PBS at a solution of 1 mM.
2. Add TAT fusion polypeptides to the media of endothelial cells at a concentration of 0, 1.0, or 10 μM .
3. Return cells to the incubator for 30 min.
4. Treat cells with 1.0 mU/mL thrombin for 30 min.
5. Analyze the cell medium for VWF with an ELISA (*see* Note 8).

3.8 Intravital Microscopy for Leukocyte Rolling

1. Anesthetize 5- to 6-week-old mice with an intramuscular injection of ketamine (80 mg/kg) and xylazine (13 mg/kg). This dose produces 30–45 min of anesthesia.
2. Inject mice intravenously with 50 μL 0.05% rhodamine 6 G to label leukocytes *in vivo*.
3. Expose the abdominal organs with a midline incision. Exteriorize the intestines on a culture dish such that the mesentery and mesenteric vessels are lying flat.
4. Place the mouse on an inverted fluorescent microscope stage with the stage warmer set at 37 °C. Select a vessel of appropriate size (~ 120 – $180 \mu\text{m}$), artery or vein. The velocity of leukocytes is slower in venules and easier to measure. Using a red fluorescent filter, fluorescent rhodamine 6G leukocytes can now be seen flowing in the vessel.
5. Collect baseline nonstimulated images prior to induction of endothelial cell exocytosis.
6. Induce endothelial exocytosis by either superfusion of a degranulating agent such as histamine (1 μM) or intravenous injection of a degranulating agent such as angiotensin.

⁸These fusion polypeptides enter cells and inhibit NSF, thus blocking exocytosis (6,7). The polypeptides contain a TAT domain and an NSF domain (10). The TAT domain is a fragment of the TAT protein from HIV that can cross cell membranes. The NSF domain is a peptide containing a sequence from NSF that inhibits NSF activity.

7. Collect images over time. Determine endothelial cell exocytosis indirectly by measuring change in leukocyte velocity from baseline or counting the number of leukocytes/frame at a given time-point normalized to the area of the imaged vessel.
8. As an alternative means to determine endothelial cell exocytosis, fluorescent 1- μ m carbamide beads can be conjugated either covalently or noncovalently to an anti-P-selectin antibody and injected into the mouse prior to stimulation. Change in microsphere velocity vs baseline can be determined as a measure of endothelial exocytosis (*see* Note 9).

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⁹Intravital microscopy is a technique used to study exocytosis in vivo. The vessels are stimulated with a compound such as histamine or FeCl₃, which triggers endothelial cell exocytosis. Exocytosis leads to the externalization of P-selectin, which mediates platelet rolling and leukocyte rolling along the vessel. Platelets or leukocytes are labeled with a fluorescent dye to visualize rolling along the vessel.

Fig. 1.

Nitric oxide inhibits endothelial cell exocytosis of VWF. HAECs were pretreated with 0.5 mM DETA-NONOate for 16 h, washed, and then stimulated with 0.5 U/mL thrombin. Media was collected at various times after thrombin stimulation and measured for VWF with an ELISA ($n = 3 \pm SD$). Some cells were pretreated with 1 mM L-mono-methylarginine (L-NMMA) for 16 h before thrombin stimulation.

Fig. 2.

NO nitrosylation of NSF is reversible. HAECs were pretreated with the NO donor SNAP (100 μ M) for 4 h and washed to remove the NO donor, and cells were harvested at various times after treatment. **(Top)** Cell lysates were immunoprecipitated with antibody to nitrosocysteine and immunoblotted with antibody to NSF. **(Bottom)** Total cell lysates were immunoblotted with antibody to NSF. (Reprinted from Ref. ⁸, © 2003, with permission from Elsevier.).

Fig. 3. Nitrosylation of NSF in vivo. **(Top)** HAECs were treated with increasing concentrations of the NOS inhibitor L-NAME. Nitrosothiols were biotinylated and precipitated with avidin-agarose; precipitants were immunoblotted with antibody to NSF. NSF is S-nitrosylated in endothelial cells, and L-NAME inhibits endogenous nitrosylation of NSF. **(Bottom)** Spleens from wild-type mice and mice lacking eNOS were harvested and prepared as above. NSF is S-nitrosylated only in mice expressing eNOS. (Reprinted from Ref. ⁸, © 2003, with permission from Elsevier.).

Fig. 4.

Antibody activates leukocyte adherence to endothelial cells. HAECs were treated with antibody to HLA (1 $\mu\text{g}/\text{mL}$) or thrombin (1 U/mL) for 45 min, and then rhodamine-6G labeled HL-60 cells were added for 15 min and then washed; adherent leukocytes were counted. Some HAECs were pretreated with antibody to P-selectin (1 $\mu\text{g}/\text{mL}$), TAT-NSF peptide (20 $\mu\text{g}/\text{mL}$), or TAT-NSFscr control peptide. (Reprinted from Ref. ¹², © 2007, National Academy of Sciences, U. S. A.).