

Hydrogen peroxide regulation of endothelial exocytosis by inhibition of *N*-ethylmaleimide sensitive factor

Kenji Matsushita,¹ Craig N. Morrell,^{2,3} Rebecca J.A. Mason,¹ Munekazu Yamakuchi,¹ Firdous A. Khanday,⁴ Kaikobad Irani,⁴ and Charles J. Lowenstein^{1,2}

¹Department of Medicine, ²Department of Pathology, and ³Department of Comparative Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205
⁴Cardiovascular Institute, University of Pittsburgh Medical Center, Pittsburgh, PA 15213

Although an excess of reactive oxygen species (ROS) can damage the vasculature, low concentrations of ROS mediate intracellular signal transduction pathways. We hypothesized that hydrogen peroxide plays a beneficial role in the vasculature by inhibiting endothelial exocytosis that would otherwise induce vascular inflammation and thrombosis. We now show that endogenous H₂O₂ inhibits thrombin-induced exocytosis of granules from endothelial cells. H₂O₂ regulates exocytosis by inhibiting *N*-ethylmaleimide sensitive factor (NSF), a protein that regulates membrane fusion

events necessary for exocytosis. H₂O₂ decreases the ability of NSF to hydrolyze adenosine triphosphate and to disassemble the soluble NSF attachment protein receptor complex. Mutation of NSF cysteine residue C264T eliminates the sensitivity of NSF to H₂O₂, suggesting that this cysteine residue is a redox sensor for NSF. Increasing endogenous H₂O₂ levels in mice decreases exocytosis and platelet rolling on venules in vivo. By inhibiting endothelial cell exocytosis, endogenous H₂O₂ may protect the vasculature from inflammation and thrombosis.

Introduction

Reactive oxygen species (ROS) play a critical role in vascular signaling, mediating cellular responses to ligands such as growth factors and cytokines (Finkel and Holbrook, 2000; Griendling et al., 2000; Xu et al., 2002). Elevated levels of ROS that are associated with cardiovascular diseases such as diabetes, hypertension, and atherosclerosis promote vascular inflammation by modulating proinflammatory transcription factors, by oxidizing LDL, and by limiting the bioavailability of nitric oxide (NO; Griendling and Alexander, 1997; Maytin et al., 1999; Harrison et al., 2003; Nedeljkovic et al., 2003). However, low levels of ROS play a physiological role by acting as second messengers (Goldschmidt-Clermont and Moldovan, 1999; Griendling and Harrison, 1999; Cai and Harrison, 2000; Finkel, 2000). Extracellular ligands activate production of intracellular ROS, which modulate specific signal transduction pathways (Sundaresan et al., 1995; Bae et al., 1997; Hampton and Orrenius, 1997; Irani et al., 1997; Ushio-

Fukai et al., 1998; Goldhaber and Qayyum, 2000; Irani, 2000; Hara et al., 2002; Liu and Gutterman, 2002; Meng et al., 2002). Low concentrations of ROS may protect the vasculature from inflammation.

Weibel-Palade body exocytosis is one mechanism by which endothelial cells promote vascular inflammation (Weibel and Palade, 1964; Wagner, 1993). Weibel-Palade bodies are endothelial cell granules that contain von Willebrand's factor (vWF) and P-selectin (Wagner et al., 1982; Larsen et al., 1989; McEver et al., 1989; Vischer and Wagner, 1993). A variety of proinflammatory agonists trigger endothelial cell exocytosis of Weibel-Palade bodies, releasing vWF into the lumen, which promotes platelet adhesion and aggregation, and translocates P-selectin to the luminal surface, which facilitates leukocyte rolling. Weibel-Palade body exocytosis is regulated by members of the SNARE superfamily and NSF (Jahn and Sudhof, 1999; Mellman and Warren, 2000; Sollner, 2003). We recently discovered that NO regulates exocytosis by *S*-nitrosylating cysteine residues on NSF (Matsushita et al., 2003). Because low levels of ROS may protect the vasculature from inflammation, we hypothesized that hydrogen peroxide modulates exocytosis by regulating NSF.

Correspondence to Charles J. Lowenstein: clowenst@jhmi.edu

Abbreviations used in this paper: 3-AT, 3-amino-triazole; HAEC, human aortic endothelial cells; NAC, *N*-acetyl-cysteine; NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; vWF, von Willebrand's factor.

Results

To explore the effect of H₂O₂ on granule exocytosis, we studied thrombin-induced exocytosis of Weibel-Palade bodies from human aortic endothelial cells (HAEC). We pretreated HAEC with H₂O₂ for 10 min, and then stimulated the cells with thrombin, and measured the amount of vWF released into the media. Thrombin induces the rapid release of vWF from HAEC (Fig. 1, A and B). However, exogenous H₂O₂ blocks the effects of thrombin in a dose-dependent manner (Fig. 1, A and B). We next examined the effect of the antioxidant *N*-acetyl-cysteine (NAC) on Weibel-Palade body exocytosis. HAEC were incubated with NAC for 4 h, washed, treated with H₂O₂, and finally stimulated with media or thrombin, and exocytosis was measured with an ELISA for vWF. NAC counteracts the inhibitory effect of H₂O₂ (Fig. 1 C).

Endogenous H₂O₂ inhibits exocytosis

We next examined the role of endogenous H₂O₂ in the regulation of Weibel-Palade body exocytosis. First, we showed that thrombin increases endogenous H₂O₂ production. We transduced HAEC with adenoviral vectors expressing β-galactosidase or catalase and measured cellular levels of H₂O₂ before and after thrombin treatment. Thrombin increases endogenous H₂O₂ production in control cells, but transduction with adenovirus-catalase blocks thrombin stimulation of endogenous H₂O₂ production (Fig. 2 A). These data suggest that thrombin

activates endogenous H₂O₂ production and catalase decreases endogenous H₂O₂ levels.

We used these adenoviral vectors to determine the effect of endogenous H₂O₂ on Weibel-Palade body exocytosis. Thrombin stimulates control HAEC to release vWF (Fig. 2 B). Expression of β-galactosidase has no effect on vWF release. However, expression of catalase increases vWF release from resting cells and from thrombin-stimulated cells (Fig. 2 B). Furthermore, expression of superoxide dismutase (SOD) decreases the release of vWF (Fig. 2 B). These data suggest that endogenous H₂O₂ produced in response to thrombin inhibits Weibel-Palade body exocytosis.

We next pretreated HAEC with angiotensin II to activate endogenous production of H₂O₂ (Ku et al., 1993; Papatropoulos et al., 1997; Dimmeler et al., 1999; Fulton et al., 1999). Treatment with 10⁻⁷ M angiotensin II for 30 min decreases thrombin-stimulated vWF release (Fig. 2 C). Catalase or an angiotensin II antagonist peptide blocks the effects of angiotensin II treatment, implying that H₂O₂ mediates angiotensin II inhibition of exocytosis (Fig. 2 C). These data suggest that endogenous H₂O₂ regulates endothelial cell exocytosis.

H₂O₂ inhibits NSF

How does H₂O₂ inhibit exocytosis? We hypothesized that H₂O₂ inhibits NSF, a protein that regulates granule exocytosis, by

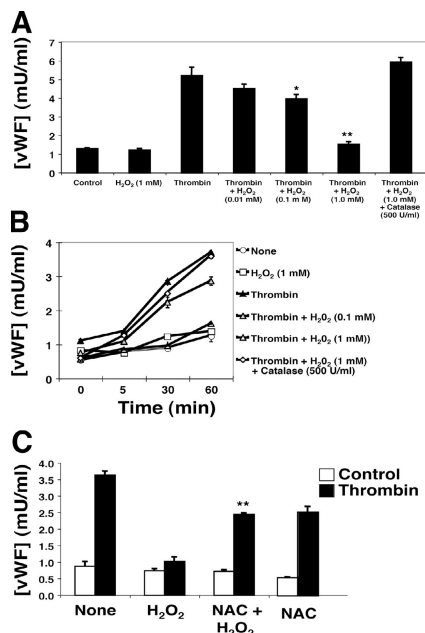


Figure 1. **Exogenous H₂O₂ inhibits exocytosis from HAEC.** (A) Dose response. HAEC were pretreated with increasing amounts of exogenous H₂O₂ for 10 min, and then treated with 1 U/ml thrombin, and the amount of vWF released into the media was measured by an ELISA ($n = 3 \pm \text{SD}$; *, $P < 0.05$ vs. thrombin; **, $P < 0.01$ vs. thrombin). (B) Time course. HAEC were pretreated with exogenous H₂O₂ for 10 min, and then treated with 1 U/ml thrombin, and the amount of vWF released into the media at increasing times was measured by an ELISA ($n = 3 \pm \text{SD}$). (C) NAC decreases exogenous H₂O₂ inhibition of thrombin-triggered vWF release. Cells were incubated with 10 mM NAC for 4 h, washed, treated with H₂O₂, and stimulated with media or thrombin, and the amount of released vWF was measured ($n = 3 \pm \text{SD}$; **, $P < 0.01$ vs. H₂O₂ alone).

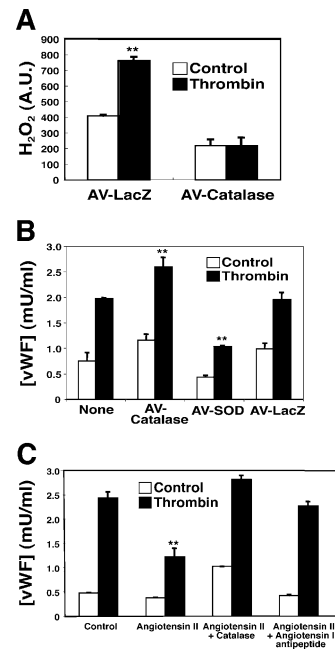


Figure 2. **Endogenous H₂O₂ inhibits exocytosis from HAEC.** (A) Catalase inhibits endogenous H₂O₂ production. HAEC were transduced with adenoviral vectors and stimulated with thrombin. The amount of H₂O₂ released from cells was measured by monitoring the increase in fluorescence of *N*-acetyl-3,7-dihydroxyphenoxazine ($n = 3 \pm \text{SD}$; **, $P < 0.01$ vs. control). (B) Endogenous H₂O₂ decreases thrombin-triggered vWF release. HAEC were transduced with adenoviral vectors and, after 48 h, treated with thrombin, and vWF was measured with an ELISA ($n = 3 \pm \text{SD}$; **, $P < 0.01$ vs. none). (C) Angiotensin II induction of endogenous H₂O₂ decreases vWF release. HAEC were stimulated with 10⁻⁷ M angiotensin II for 30 min, and then incubated with thrombin and 500 U/ml catalase or 10 mM angiotensin II antipeptide. The amount of vWF released from cells into the media was measured by an ELISA ($n = 3 \pm \text{SD}$; **, $P < 0.01$ vs. control).

hydrolyzing ATP and by interacting with SNARE molecules (Block et al., 1988; Malhotra et al., 1988; Mellman and Warren, 2000). We first examined the effect of H₂O₂ on the ATPase activity of NSF, which is critical for NSF function (Whiteheart et al., 1994). H₂O₂ was added to 10 μg of recombinant NSF, and the ATPase activity of NSF was measured by a colorimetric assay. H₂O₂ significantly inhibits NSF hydrolysis of ATP (Fig. 3 A).

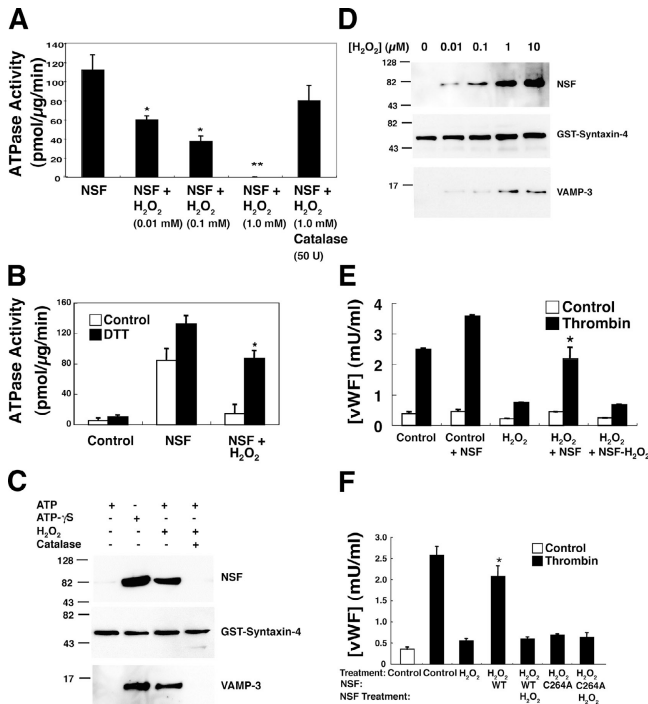


Figure 3. H₂O₂ inhibits NSF. (A) H₂O₂ inhibits ATPase activity of wild-type NSF. H₂O₂ or control was added to recombinant wild-type NSF, and the ATPase activity of NSF was measured ($n = 2 \pm \text{SD}$; *, $P < 0.05$ vs. NSF; **, $P < 0.01$ vs. NSF). (B) DTT restores ATPase activity of wild-type NSF inhibited by H₂O₂. H₂O₂ or control was added to recombinant wild-type NSF, buffer or 1 mM DTT was added, and the ATPase activity of NSF was measured ($n = 3 \pm \text{SD}$; *, $P < 0.01$ for H₂O₂ vs. H₂O₂ + DTT). (C) H₂O₂ inhibits disassembly activity of wild-type NSF. Recombinant (His)₆-NSF was pretreated or not with 1 mM H₂O₂ and incubated with (His)₆-α-SNAP, GST-Syntaxin-4, VAMP-3, and SNAP-23. ATP or ATP-γS was added, and the mixture was precipitated with glutathione-sepharose. Precipitated proteins were immunoblotted with antibody to the NSF tag (top), to syntaxin-4 (middle), or to VAMP-3 (bottom). Experiment was repeated three times with similar results. (D) H₂O₂ inhibits disassembly activity of wild-type NSF (dose response). The NSF disassembly assay was performed, pretreating recombinant (His)₆-NSF with increasing concentrations of H₂O₂ and then mixing with (His)₆-α-SNAP, GST-Syntaxin-4, VAMP-3, and SNAP-23. Proteins precipitated with glutathione-sepharose were immunoblotted with antibody to the NSF tag (top), to syntaxin-4 (middle), or to VAMP-3 (bottom). Experiment was repeated three times with similar results. (E) Exogenous NSF restores vWF exocytosis in endothelial cells treated with H₂O₂. HAEC were pretreated with 1 mM H₂O₂ for 10 min, permeabilized with SLO, incubated with recombinant NSF or H₂O₂-treated recombinant NSF, and stimulated with thrombin, and the amount of vWF in the media was measured ($n = 3 \pm \text{SD}$; *, $P < 0.01$ for H₂O₂ vs. H₂O₂ + NSF). (F) NSF mutant C264A does not restore exocytosis. HAEC were pretreated with 1 mM H₂O₂ for 10 min, permeabilized, and incubated with recombinant wild-type NSF (WT) or mutant NSF(C264A). In some cases, the recombinant NSF was treated with H₂O₂ before addition to cells. The cells were then resealed and stimulated with thrombin, and the amount of vWF in the media was measured ($n = 3 \pm \text{SD}$; *, $P < 0.01$ vs. H₂O₂ + Thrombin).

If H₂O₂ reversibly oxidizes NSF cysteine residues, then the reducing agent DTT would be predicted to reduce oxidized cysteine residues and restore NSF ATPase activity. To test this prediction, we treated recombinant NSF with H₂O₂, added DTT, and measured the ATPase activity of NSF. H₂O₂ inhibits NSF ATPase activity, and DTT restores ATPase activity of NSF exposed to H₂O₂ (Fig. 3 B).

We next explored the effect of H₂O₂ on NSF disassembly activity. NSF interacts with SNARE molecules using α-SNAP as an adaptor (Jahn and Sudhof, 1999; Mellman and Warren, 2000). ATP-γS locks NSF onto the SNARE complex; however, ATP enables NSF to separate from and disassemble the SNARE complex. Accordingly, we examined the effect of H₂O₂ on NSF disassembly of recombinant SNARE molecules. Recombinant (His)₆-NSF was pretreated or not with H₂O₂. Then, (His)₆-NSF and (His)₆-α-SNAP were incubated with recombinant SNARE polypeptides that regulate Weibel-Palade body exocytosis: GST-syntaxin-4 as well as nontagged VAMP-3 and SNAP-23. ATP or ATP-γS was added to the mixture, the mixture was precipitated with glutathione-sepharose beads, and precipitated proteins were fractionated by SDS-PAGE and immunoblotted with antibody to the NSF tag, syntaxin, and VAMP-3.

ATP-γS increases the interaction of NSF with SNARE polypeptides. ATP decreases the interaction of NSF with SNARE polypeptides (Fig. 3 C). However, H₂O₂ blocks NSF disassembly of the SNARE complex in the presence of ATP (Fig. 3 C). H₂O₂ inhibits disassembly activity of wild-type NSF in a dose-dependent manner (Fig. 3 D). Together, these data show that H₂O₂ blocks NSF disassembly activity.

We next confirmed that NSF is an intracellular target of H₂O₂. We treated HAEC with H₂O₂, and then permeabilized the cells and added recombinant NSF. H₂O₂ blocks exocytosis as before (Fig. 3 E). Recombinant NSF restores exocytosis to cells inhibited with H₂O₂ (Fig. 3 E). Furthermore, oxidized NSF cannot restore exocytosis to HAEC (Fig. 3 E). As an additional control, we added to HAEC either wild-type NSF or a mutant NSF(C264A) with decreased ATPase activity. Although wild-type NSF restores secretion to endothelial cells, the kinase-dead mutant NSF does not (Fig. 3 F). These data demonstrate that NSF is an intracellular target of H₂O₂.

Specific cysteine residues mediate NSF sensitivity to H₂O₂

H₂O₂ may regulate NSF by oxidizing cysteine residues. To determine which of the nine cysteine residues of NSF are targets of H₂O₂, we expressed in bacteria and purified wild-type or mutant NSF polypeptides with each of the nine individual cysteine residues of NSF replaced by Ala. We added H₂O₂ to wild-type and mutant NSF polypeptides and measured ATPase activity. Mutation of cysteine residues 21, 91, 264, and 334 decreases NSF ATPase activity (Fig. 4 A). H₂O₂ treatment inhibits ATPase activity of all mutant NSF except mutants C21A and C264A (Fig. 4 A). These data suggest that cysteine residues C21 and C264 mediate H₂O₂ inhibition of NSF ATPase activity.

We next determined which cysteine residues mediate H₂O₂ inhibition of NSF separation from the SNARE complex.

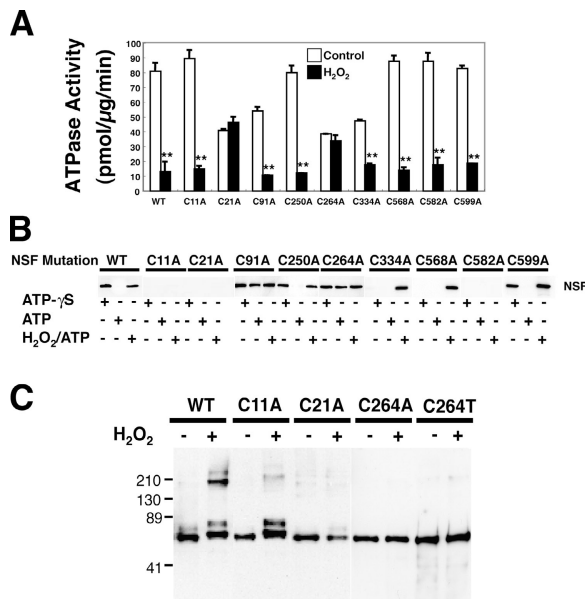


Figure 4. NSF cysteine residue targets of H₂O₂. (A) Specific NSF mutants are resistant to H₂O₂ inhibition of ATPase activity. 1 mM H₂O₂ or control was added to recombinant wild-type and mutant NSF, and the ATPase activity was measured. H₂O₂ inhibits ATPase activity of wild-type NSF and all mutant NSF, except for C21A and C264A mutants ($n = 2 \pm \text{SD}$; **, $P < 0.01$ vs. control). (B) H₂O₂ does not affect interaction of specific NSF mutants with syntaxin-4. (His)₆-NSF was pretreated or not with 1 mM H₂O₂, and then mixed with (His)₆- α -SNAP, GST-Syntaxin-4, VAMP-3, and SNAP-23. The mixture was precipitated with glutathione-sepharose and immunoblotted with antibody to NSF. (C) H₂O₂ alters the mobility of NSF mutants. Recombinant wild-type or mutant NSF was treated with 0.1 mM H₂O₂ for 5 min, fractionated by nondenaturing PAGE, and then immunoblotted with antibody to NSF.

We used the NSF-SNARE pull-down assay, adding H₂O₂ to NSF mutants lacking individual cysteine residues, along with α -SNAP, GST-syntaxin-4, VAMP-3, and SNAP-23. H₂O₂ blocks the ability of wild-type NSF to separate from the SNARE complex in the presence of ATP (Fig. 4 B). Mutation of cysteine residues 250 and 599 has no effect on the ability of H₂O₂ to inhibit NSF separation from the SNARE complex. The effect of H₂O₂ on cysteine residues 11, 21, 334, 568, and 582 cannot be ascertained because mutation of these residues abrogates NSF interaction with SNARE molecules. Mutation of cysteine residues 91 and 264 blocks the ability of NSF to separate from the SNARE complex, and H₂O₂ has no effect on these mutants. These data exclude cysteine residues C250 and C599 as targets of H₂O₂, and the data indirectly suggest that cysteine residues C91 and C264 may mediate H₂O₂ inhibition of NSF separation from the SNARE complex.

To explore the physical effects of H₂O₂ on NSF, we exposed recombinant wild-type and mutant NSF to H₂O₂, fractionated the NSF by nondenaturing PAGE, and then immunoblotted with antibody to NSF. Wild-type NSF runs as two bands, a darker band at ~ 70 kD and a fainter band at ~ 85 kD (Fig. 4 C). H₂O₂ changes the mobility of wild-type NSF, generating a doublet of decreased mobility (Fig. 4 C). We next determined which cysteine residues mediate the shift in mobility induced by H₂O₂. We treated mutant NSF with H₂O₂ and then examined the mobility on nondenaturing PAGE. Oxidant stress causes a

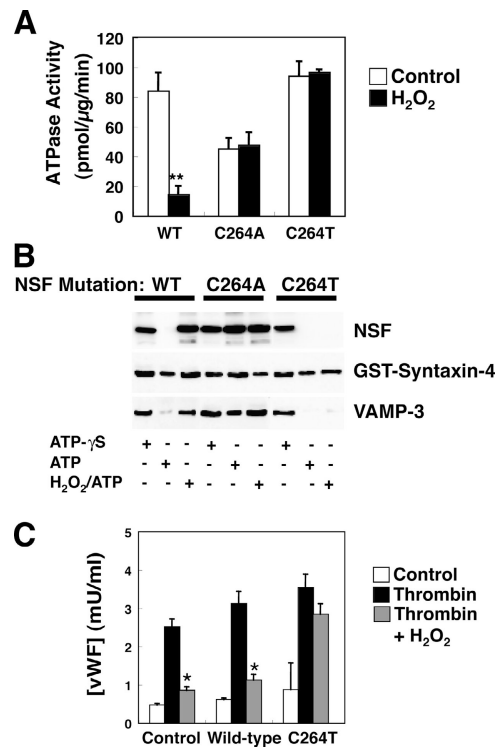


Figure 5. Mutant NSF(C264T) is resistant to H₂O₂. (A) H₂O₂ does not affect ATPase activity of mutant NSF(C264T). The ATPase assay was performed, adding recombinant wild-type or mutant NSF. 100 μM H₂O₂ inhibits ATPase activity of wild-type NSF but not of mutant NSF(C264T). H₂O₂ has no effect on ATPase activity of NSF(C264A) ($n = 3 \pm \text{SD}$; **, $P < 0.01$ vs. control). (B) H₂O₂ does not affect disassembly activity of specific NSF mutants. The disassembly assay was performed, adding α -SNAP, GST-Syntaxin-4, VAMP-3, SNAP-23, and recombinant wild-type NSF (WT) or mutant NSF(C264A) or NSF(C264T). Precipitated proteins were immunoblotted with antibody to the NSF tag (top), to syntaxin-4 (middle), or to VAMP-3 (bottom). Experiment was repeated three times with similar results. 1 mM H₂O₂ inhibits disassembly activity of wild-type NSF but not of mutant NSF(C264T). (C) H₂O₂ does not affect exocytosis in endothelial cells containing NSF mutants. HAEC were permeabilized with SLO and incubated with recombinant wild-type NSF or mutant NSF(C264T). Cells were resealed, pretreated with 0 or 100 μM H₂O₂ for 10 min, and treated with thrombin. The amount of vWF released into the media was measured by an ELISA ($n = 3 \pm \text{SD}$; *, $P < 0.01$ for thrombin vs. thrombin + H₂O₂).

slight decrease in mobility of NSF(C11A) and NSF(C21A). In contrast, H₂O₂ does not change the mobility of NSF(C264A) (Fig. 4 C). These data suggest that H₂O₂ alters the physical properties of NSF and that C264 mediates some of the physical effects of H₂O₂ on NSF.

Mutant NSF(C264T) is resistant to H₂O₂

We next constructed a H₂O₂-resistant NSF mutant and used it to make an endothelial cell line containing H₂O₂-resistant NSF. Our data suggested that C264 may be a redox-sensitive cysteine residue in NSF. A comparison of primary NSF amino acid sequences reveals that C264 in the Walker A box of the D1 domain of NSF may be a recent evolutionary adaptation (Sollner and Sequeira, 2003). The amino acid residue in this position is cysteine in vertebrates but is threonine in insects, plants, and yeast (Sollner and Sequeira, 2003). We hypothe-

sized that a mutant NSF(C264T) would retain NSF activity but would be resistant to H₂O₂. To test this idea, we constructed the NSF mutant NSF(C264T).

We first compared the effect of H₂O₂ on the ATPase activity of recombinant wild-type NSF and mutant NSF(C264T). The ATPase activity of the NSF(C264T) mutant is approximately the same as that of wild-type NSF, although the ATPase activity of mutant NSF(C264A) is greatly decreased (Fig. 5 A). H₂O₂ inhibits ATPase activity of wild-type NSF but not of mutant NSF(C264T) (Fig. 5 A).

We next compared the effect of H₂O₂ on the disassembly activity of wild-type NSF and mutant NSF(C264T). The disassembly activity of the NSF(C264T) mutant is similar to that of the wild-type NSF (Fig. 5 B). H₂O₂ inhibits disassembly activity of wild-type NSF but not of mutant NSF(C264T) (Fig. 5 B).

We then constructed endothelial cells that contain the NSF(C264T) mutant. Endothelial cells were permeabilized with SLO and then incubated with wild-type or mutant NSF. Thrombin activates exocytosis in HAEC containing wild-type NSF and mutant NSF(C264T) (Fig. 5 C). H₂O₂ inhibits exocytosis from cells containing wild-type NSF. In contrast, H₂O₂ does not affect exocytosis from endothelial cells containing NSF(C264T) (Fig. 5 C). Together, these data show that NSF residue C264 is a target of H₂O₂. These data also support the hypothesis that H₂O₂ inhibits exocytosis by oxidation of NSF.

H₂O₂ inhibits exocytosis in vivo

We also examined the physiological effects of H₂O₂ on exocytosis in vivo. If H₂O₂ inhibits exocytosis, then we would expect catalase inhibitors to increase endogenous H₂O₂ levels and to decrease endothelial release of vWF. We first tested this hypothesis in endothelial cells with the catalase inhibitor 3-aminotriazole (3-AT). Increasing doses of 3-AT increase endothelial levels of H₂O₂ (Fig. 6 A). Increasing doses of 3-AT also block endothelial exocytosis (Fig. 6 B). We examined this phenomenon in mice. We administered 3-AT to mice, and examined H₂O₂ levels and exocytosis after 5 h. The catalase inhibitor 3-AT increases H₂O₂ levels in murine liver (Fig. 6 C). We examined the effect of 3-AT on platelet rolling along murine venules stimulated with FeCl₃; platelet rolling is mediated in part by vWF released by endothelial exocytosis of Weibel-Palade bodies (Andre et al., 2000). Mice were treated with 3-AT or PBS, anesthetized, and injected with calcein-AM-labeled platelets. The mesentery was externalized, endothelial exocytosis was induced by superfusing with FeCl₃, and platelet rolling on mesenteric venules was recorded using a digital fluorescent camera. FeCl₃ activates platelet rolling in control mice (Fig. 6, D and E). However, 3-AT greatly inhibits FeCl₃-activated platelet rolling in mice (Fig. 6, D and E). Together, these data suggest that H₂O₂ regulates endothelial exocytosis in vivo.

Discussion

The major finding of our study is that H₂O₂ regulates exocytosis by inhibiting NSF. Construction of an H₂O₂-resistant mutant NSF suggests that NSF residue C264 serves as a redox sensor for NSF. These data extend our report that NO regulates

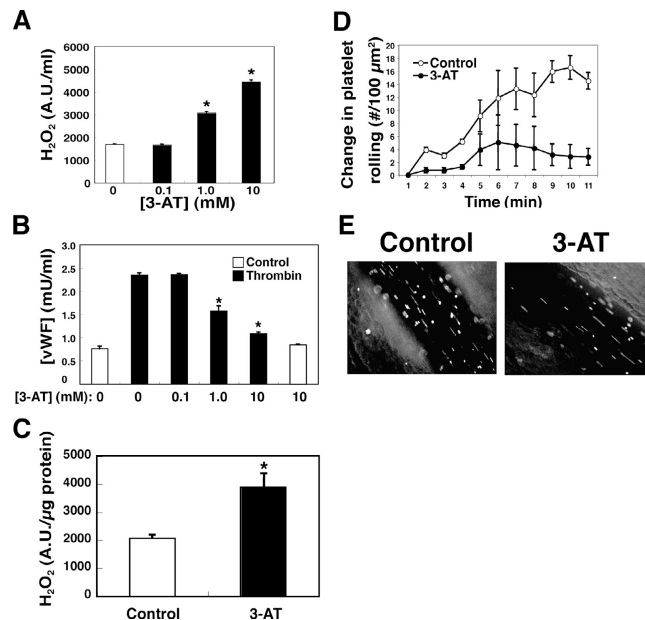


Figure 6. Hydrogen peroxide inhibits vWF release in vivo. (A) The catalase inhibitor 3-AT increases H₂O₂ in cells. HAEC were pretreated with 3-AT for 2 h. The amount of H₂O₂ released from cells was measured by monitoring the increase in fluorescence of *N*-acetyl-3,7-dihydroxyphenoxazine ($n = 3 \pm \text{SD}$; *, $P < 0.01$ vs. 0 mM). (B) The catalase inhibitor 3-AT decreases thrombin-induced exocytosis. HAEC were pretreated with 3-AT for 2 h, and then incubated with thrombin for 1 h. The amount of vWF released from cells into the media was measured by an ELISA ($n = 3 \pm \text{SD}$; *, $P < 0.01$ vs. thrombin alone). (C) The catalase inhibitor 3-AT increases H₂O₂ in mice. Mice were pretreated with 500 mg/kg 3-AT i.p. After 5 h, the amount of H₂O₂ released from spleen homogenates was measured by monitoring the increase in fluorescence of *N*-acetyl-3,7-dihydroxyphenoxazine ($n = 3 \pm \text{SD}$; *, $P < 0.01$ vs. control). (D) The catalase inhibitor 3-AT decreases platelet rolling in mice. Mice were pretreated or not with 500 mg/kg 3-AT, and after 5 h the mice were anesthetized and injected with calcein-AM-labeled platelets from nontreated mice. The mesentery was externalized, and 120–150- μm -diam venules were treated with 1 mM histamine. Platelet rolling on venules was imaged with a digital fluorescent camera ($n = 5–6 \pm \text{SEM}$). (E) Digital fluorescence images of platelet rolling in mice treated with control or 3-AT (representative images from $n = 5–6$).

exocytosis by nitrosylating NSF (Matsushita et al., 2003). NO and H₂O₂ appear to have distinct effects on NSF. Both NO and H₂O₂ inhibit NSF disassembly activity, but only H₂O₂ inhibits NSF ATPase activity. Together, our data suggest that the intracellular redox state regulates exocytosis.

Thrombin not only stimulates endothelial exocytosis but also stimulates H₂O₂ production, which inhibits exocytosis. Other compounds that activate endothelial exocytosis also increase H₂O₂ production, such as epinephrine, VEGF, and ceramide (Griendling and Alexander, 1997; Goldschmidt-Clermont and Moldovan, 1999; Finkel, 2001). H₂O₂ may thus serve as a negative feedback signal to regulate exocytosis. An imbalance in oxidant stress (e.g., an increase in oxidants or a decrease in antioxidant levels) would be predicted to decrease exocytosis, limiting thrombosis and inflammation in the vasculature.

Several large randomized clinical trials have demonstrated that antioxidants do not reduce mortality or cardiovascular outcomes (Virtamo et al., 1998; Yusuf et al., 2000; de Gaetano, 2001; Heart Protection Study Collaborative Group,

2002; Morris and Carson, 2003). Our data suggest one explanation for this lack of benefit: endogenous oxidants protect the vasculature by inhibiting endothelial exocytosis that would otherwise lead to vascular inflammation and thrombosis. Future therapies aimed at modulating endogenous oxidants may have to be narrowly tailored to block the harmful effects of radicals while preserving the beneficial effects.

Materials and methods

Materials

Thrombin was purchased from Enzyme Research Laboratories. H_2O_2 , catalase, NAC, and angiotensin II were purchased from Sigma-Aldrich. Mouse mAbs to NSF and syntaxin-4 were purchased from BD Biosciences. The cDNA for GST-syntaxin-4, GST-SNAP-23, and GST-VAMP-3 were provided by J. Pevsner (Johns Hopkins University School of Medicine, Baltimore, MD). The cDNAs of RGS-His₆-NSF and RGS-His₆- α -SNAP were gifts from J.E. Rothman (Rockefeller University, New York, NY).

Preparation of recombinant adenoviruses

The replication-deficient adenovirus encoding the epitope-tagged catalase, SOD cDNA, and the adenovirus-LacZ were constructed by homologous recombination in 293 cells with use of the adenovirus-based plasmid JM17 as previously described (Sundaresan et al., 1995; Irani et al., 1997). All viruses were amplified and titered in 293 cells and purified on CsCl gradients.

Preparation of recombinant NSF and SNARE polypeptides

Mutation of the cysteine residues to alanine residues of NSF was performed with a kit according to the manufacturer's instructions (Stratagene). Recombinant RGS-(His)₆-NSF and RGS-(His)₆- α -SNAP were expressed in bacteria and purified on a Ni-NTA-agarose column (HisTRAP; Amersham Biosciences). Recombinant GST-SNARE proteins were expressed in BL21 cells and purified with glutathione-agarose (GSTrap; Amersham Biosciences). For some assays, the GST tag was cleaved off of the GST-SNARE polypeptides GST-VAMP-3 and GST-SNAP-23 with thrombin.

Cell culture and analysis of vWF release

HAEC were obtained from Clonetics and grown in EGM-2 media (Clonetics). To measure the effect of H_2O_2 on vWF release, HAEC were pretreated with H_2O_2 for 10 min in the presence or absence of catalase. The cells were washed and stimulated with 1 U/ml thrombin, and the amount of vWF released into the media was measured by an ELISA (American Diagnostica, Inc.). To measure the effect of endogenous H_2O_2 on exocytosis, HAEC were pretreated for 48 h with 200 MOI of adenovirus-catalase, adenovirus-SOD, and adenovirus-LacZ before thrombin stimulation. As an alternative approach, HAEC were pretreated with 10 mM NAC for 4 h, washed with EGM-2 medium, and stimulated with 1 U/ml thrombin. The supernatants were collected, and the concentration of vWF released into the media was measured by an ELISA.

Permeabilization of HAEC

To determine the role of NSF and NSF mutants in Weibel-Palade body exocytosis, HAEC were permeabilized, incubated with recombinant NSF or NSF mutant polypeptides, and resealed. We developed a permeabilization protocol specific for endothelial cells by following a method for optimization of permeabilization with SLO (Walev et al., 2001). To permeabilize HAEC, cells were grown in 96-well plates, washed with HBSS without Mg^{2+} and Ca^{2+} , and incubated for 15 min at 37°C with 10 U SLO in 50 μ l PBS, pH 7.4, along with 100 μ g/ml NSF or NSF mutants. Cells were then resealed by incubation with 250 μ l EGM-2 medium containing 2% FBS for 4 h at 37°C. The HAEC were then washed with EGM-2 medium and stimulated with thrombin. The supernatants were collected and the concentration of vWF released into the media was measured by an ELISA.

Endothelial H_2O_2 production

H_2O_2 was quantified using the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes) according to the manufacturer's recommendations. The fluorescence of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex red reagent), a highly sensitive and stable probe for H_2O_2 , was measured with a Cytofluor 2300 fluorimeter (Millipore; Mohanty et al., 1997). Values represent the mean \pm SD from a representative experiment that was repeated twice.

ATPase assay

The ATPase activity of NSF was measured by a coupled assay in which ATP utilization is linked to the pyruvate kinase reaction, which generates pyruvate, which in turn is measured continuously with lactate dehydrogenase (Huang and Hackney, 1994). Recombinant NSF (0.2 μ g/ μ l) was pretreated with buffer or H_2O_2 for 10 min at 22°C. ATPase reaction buffer (100 mM Hepes buffer, pH 7.0, 100 mM KCl, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM ATP, 5 mM phosphoenol pyruvate, 50 U lactate dehydrogenase, and 50 U pyruvate kinase) was added to the mixture, followed by 10 μ l of NADH (2 mg/ml in 1% sodium bicarbonate). The mixture was incubated for 10 min at 22°C, and the absorbance was measured at 340 nm.

NSF disassembly assay

The disassembly activity of NSF was measured by a coprecipitation assay as described previously (Pevsner et al., 1994; Matsushita et al., 2003). Recombinant RGS-(His)₆-NSF (0.1 μ g/ μ l) was pretreated with buffer or H_2O_2 for 10 min at 22°C. Recombinant RGS-(His)₆- α -SNAP (0.1 μ g/ μ l) and SNARE polypeptides (0.1 μ g/ μ l each of VAMP-3, SNAP-23, and GST-Syntaxin-4) were added, followed by either 2.5 mM ATP/5 mM $MgCl_2$ or 2.5 mM ATP- γ S/5 mM $MgCl_2$. This mixture of NSF and SNARE polypeptides was then incubated in binding buffer (4 mM Hepes, pH 7.4, 0.1 M NaCl, 1 mM EDTA, 3.5 mM $CaCl_2$, 3.5 mM $MgCl_2$, and 0.5% NP-40) and glutathione-sepharose beads for 1 h at 4°C with rotation. The beads were washed with binding buffer four times, mixed with SDS-PAGE sample buffer, boiled for 3 min, and analyzed by immunoblotting.

The ability of NSF to separate from SNARE polypeptides was measured using a similar assay. Recombinant RGS-(His)₆-NSF was incubated with recombinant RGS-(His)₆- α -SNAP (0.1 μ g/ μ l) and SNARE polypeptides (0.1 μ g/ μ l each of GST-Syntaxin-4, VAMP-3, and SNAP-23). The GST-tagged syntaxin-4 fusion polypeptide was precipitated with glutathione-sepharose beads, and the ability of NSF to separate from syntaxin-4 was measured by immunoblotting precipitants for NSF.

Intravital microscopy

Intravital microscopy was performed as has been previously described (Andre et al., 2000). Platelets were isolated and purified from wild-type C57BL6/J mice (The Jackson Laboratory) and incubated for 20 min with 1 μ M calcein-AM (Molecular Probes). Wild-type mice were pretreated with saline or 3-AT for 5 h, anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg), and then injected i.v. with 5×10^7 platelets for the rolling study or 10^8 platelets for the thrombosis study. The mesentery was exteriorized and 120–150- μ m-diam venules were selected, and the mouse mesentery was prepared on a stage heated to 37°C of an inverted fluorescent microscope (model Eclipse TE200; Nikon). Endothelial damage was induced by superfusion of 1 mM histamine, and images of platelet rolling were captured with a digital camera (Retiga Exi Fast1394; QImaging) through a Modulation Optics objective lens with a 20 \times magnification. The images were collected by QCapture PRO imaging software (QImaging) and imported into Adobe Photoshop Creative Suites on an Apple PowerPC G4 computer. Each image was adjusted with Adobe Photoshop CS by selecting the entire image, opening the Levels dialog box, dragging the black Input Level slider to the leftmost cluster of pixels in the histogram, and dragging the white Input Level slider to the rightmost cluster of pixels in the histogram. Platelet rolling was determined by counting the number of platelets that remained transiently within a frame for the 30-ms collection time.

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