

S-Nitrosation of β -Catenin and p120 Catenin A Novel Regulatory Mechanism in Endothelial Hyperpermeability

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Rationale: Endothelial adherens junction proteins constitute an important element in the control of microvascular permeability. Platelet-activating factor (PAF) increases permeability to macromolecules via translocation of endothelial nitric oxide synthase (eNOS) to cytosol and stimulation of eNOS-derived nitric oxide signaling cascade. The mechanisms by which nitric oxide signaling regulates permeability at adherens junctions are still incompletely understood.

Objective: We explored the hypothesis that PAF stimulates hyperpermeability via S-nitrosation (SNO) of adherens junction proteins.

Methods and Results: We measured PAF-stimulated SNO of β -catenin and p120-catenin (p120) in 3 cell lines: ECV-eNOSGFP, EAhy926 (derived from human umbilical vein), and postcapillary venular endothelial cells (derived from bovine heart endothelium) and in the mouse cremaster muscle in vivo. SNO correlated with diminished abundance of β -catenin and p120 at the adherens junction and with hyperpermeability. Tumor necrosis factor- α increased nitric oxide production and caused similar increase in SNO as PAF. To ascertain the importance of eNOS subcellular location in this process, we used ECV-304 cells transfected with cytosolic eNOS (GFPeNOSG2A) and plasma membrane eNOS (GFPeNOSCAAX). PAF induced SNO of β -catenin and p120 and significantly diminished association between these proteins in cells with cytosolic eNOS but not in cells wherein eNOS is anchored to the cell membrane. Inhibitors of nitric oxide production and of SNO blocked PAF-induced SNO and hyperpermeability, whereas inhibition of the cGMP pathway had no effect. Mass spectrometry analysis of purified p120 identified cysteine 579 as the main S-nitrosated residue in the region that putatively interacts with vascular endothelial-cadherin.

Conclusions: Our results demonstrate that agonist-induced SNO contributes to junctional membrane protein changes that enhance endothelial permeability. (*Circ Res.* 2012;111:553-563.)

Key Words: S-nitrosation ■ adherens junction ■ microvascular permeability ■ nitric oxide ■ p120

Cell life depends on an adequate supply of nutrients and removal of catabolites via blood circulation. This important exchange function is controlled mainly by the microvascular endothelium. Inflammation and wound healing require specific regulation of microvascular permeability to macromolecules to enable tissue repair. The regulation of permeability occurs primarily at postcapillary venules in vivo through activation of endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO), which has been identified as a key signaling element in eliciting hyperperme-

ability.¹⁻⁵ The mechanisms by which eNOS-derived NO induces hyperpermeability include the internalization of eNOS indicating that eNOS location and exact delivery of NO regulate the development of hyperpermeability.^{2,6}

Endothelial cells from postcapillary venules display adherens junctions, which contain vascular endothelial (VE)-cadherin, forming a complex with the cytosolic proteins α -catenin, β -catenin, plakoglobin, and p120-catenin (p120).⁷⁻¹¹ Proinflammatory agents stimulate signaling cascades that by phosphorylation of adherens junction

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Non-standard Abbreviations and Acronyms

| | |
|--------------------------------|---|
| CVEC | bovine coronary venular postcapillary endothelial cells |
| eNOS | endothelial nitric oxide synthase |
| L-NMA | L-NG-methyl-L-arginine |
| NAC | N-acetyl-L-cysteine |
| NO | nitric oxide |
| ODQ | 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one |
| PAF | platelet activating factor |
| PKG | protein kinase G |
| sGC | soluble guanylate cyclase |
| SNO | S-nitrosation |
| TNF-α | Tumor necrosis factor α |
| VE | vascular endothelial |

proteins lead to their internalization, which results in increased endothelial paracellular permeability.^{12,13}

Despite advances in the research of hyperpermeability regulation, the mechanisms by which NO signaling influences processes occurring at the adherens junctions remain unknown. The classic dogma in NO signaling establishes that all of the actions of NO are mediated via soluble guanylate cyclase (sGC) and protein kinase G (PKG). However, increases in permeability have been described in some cellular models regardless of PKG activation but still requiring NO production.¹⁴ Recently, S-nitrosation (SNO) has emerged as an important NO-dependent posttranslational modification of free-thiol cysteines that alters the function of proteins and requires proximity between eNOS and the target proteins for appropriate NO delivery.^{15–17} We report here experiments testing the hypothesis that NO regulates endothelial permeability to macromolecules by SNO of β -catenin and p120, 2 important and integral components of the adherens junction. We determined that platelet-activating factor (PAF) induces SNO of β -catenin and p120 and their internalization away from the plasma membrane. These processes are associated with the onset of hyperpermeability. Furthermore, we demonstrate that eNOS located in the cytosol, but not the plasma membrane-anchored eNOS, causes SNO of β -catenin and p120. This advance in knowledge may serve as a basis for the development of therapeutic agents in the treatment of vascular diseases having a phase characterized by inflammation (eg, ischemia-reperfusion injury, stroke, and atherosclerosis).

Methods

An expanded Methods section is provided in the online-only Data Supplement.

Reagents

PAF and N-acetyl-L-cysteine (NAC) were obtained from Calbiochem. FITC-labeled dextran 70 (FITC-Dx-70; molecular weight: 70 000 Da), Acetylcholine, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), N-ethylmaleimide, and NG-methyl-L-arginine (L-NMA) were from Sigma. Tumor necrosis factor α (TNF- α) was from Roche.

Antibodies

Mouse anti-p120 catenin was from BD Transduction Laboratories. Rabbit anti- β -catenin and mouse anti- β -actin, were from Sigma.

Plasmids and Cell Transfections

GFPeNOS-G2A and GFPeNOS-CAAX constructs were kindly provided by Dr. David Fulton (Georgia Health Sciences University, Augusta, GA). ECV-304 cells were transfected with fluorescently labeled eNOS mutant constructs using Lipofectamine according to manufacturers' instructions (Invitrogen). Transfected cells were selected for growth in medium including 1 mg/mL of geneticin (G418) (Invitrogen). Resistant colonies were maintained in the complete Dulbecco's modified Eagle's medium containing G418 (400 μ g/mL).

Cell Culture

ECV-304, ECV-eNOSGFP, ECV-GFPeNOS-G2A, ECV-GFPeNOS-CAAX, EAhy296, and postcapillary venular endothelial cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin. ECV-eNOSGFP, ECVG2A-eNOSGFP, and ECVCAAX-eNOSGFP were additionally supplemented with geneticin (G418) 400 μ g/mL. ECV-304 and ECV-eNOSGFP were kindly donated by Dr. William Sessa (Yale University, New Haven, CT). EAhy296 cells were kindly provided by Dr C. J. S. Edgell (University of North Carolina, Chapel Hill, NC).

Immunoprecipitation

Cell lysates from control and agonist-treated cells were incubated with specific antibodies overnight at 4°C. Protein A/G beads were added to samples for 2 hours at 4°C and then pelleted by centrifugation and washed with lysis buffer. Proteins of interest were detected using Western blotting and chemiluminescence. Quantification of changes from control was evaluated by densitometric analysis of Western blots using the NIH Image J Program.

Biotin-Switch Assay

Proteins were denatured with sodium dodecyl sulfate in the presence of methyl methanethiosulfonate.¹⁸ After acetone precipitation to remove excess methyl methanethiosulfonate, 1 mmol/L ascorbate and biotin- N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide were added to reduce the SNO bond and label the reduced thiol with biotin, respectively. Biotinylated proteins were captured with streptavidin-agarose beads and then separated by sodium dodecyl sulfate-PAGE and detected with specific antibodies. The biotin-switch assay was performed on 100 μ g of lysates from control and agonist-treated cells. Proteins of interest were detected by Western blotting.

Immunofluorescence Microscopy

We followed established protocols.^{19,20} Cells were cultured on glass coverslips and treated with agonist and then fixed and permeabilized in 100% ethanol for 10 minutes at –20°C. The secondary Alexa Fluor conjugated antibodies were added after incubation with corresponding primary antibodies for 1 hour at room temperature. Coverslips were mounted and visualized by fluorescence microscopy. Images were obtained using an epifluorescence microscope (Axioscop; Carl Zeiss) equipped with a 100 \times oil immersion objective lens and Axio Vision Rel software (Zeiss, Germany). Eight-bit images were prepared for illustration in Photoshop (Adobe).

Endothelial Permeability Assay

Monolayer permeability was determined as described previously.^{2,21} Cells were grown on fibronectin-coated polycarbonate membranes,

for 5 to 6 days to achieve confluence. On the day of the experiment, the membranes were placed in a Navicte system (San Diego, CA). Luminal and abluminal chambers were filled with Dulbecco's modified Eagle's medium without phenol red. After a 15-minute equilibration period, the luminal chamber was loaded with FITC-Dx-70 (final concentration 13.3 mg/mL). Samples for baseline permeability were obtained every 5 minutes for a period of 30 minutes. After addition of PAF, samples were obtained for an additional 30-minute period. In the experiments using NAC (2.5 mmol/L), this agent was added for 1.5 hour to the cells in the plates prior to the addition of agonists. Fresh NAC was applied for 1 more hour to the cells when they were transferred to the Navicte system. In the experiments using L-NMA (300 μ mol/L), the NOS inhibitor was added for 1 hour while the cells were in the Navicte system before PAF addition. The permeability to FITC-Dx-70 was determined according to the Fick equation. FITC-Dx-70 was measured using a spectrofluorometer.

In Vivo Cremaster Preparation

Male wild-type (C57BL/6J; Jackson Laboratory, Bar Harbor, MA) were anesthetized with Ketamine (90 mg/Kg)-Xylazine (10 mg/Kg). The cremaster muscle was exposed via a scrotal incision and gently separated from the subcutaneous interstitial tissue under continuous superfusion with intravital buffer solution equilibrated with 95% N₂ plus 5% CO₂, pH 7.4, 35°C. The whole cremaster sac, containing the testis, was positioned in a plastic container and superfused. After 15 minutes equilibration, 10⁻⁷ mol/L PAF was applied topically, bathing the whole muscle for 3 minutes. The cremaster and the testis were ligated and quickly excised. The muscle was immediately separated from the testis and homogenized in 500 μ L buffer containing an antiprotease cocktail. The contralateral cremaster was treated similarly, except that buffer was applied instead of PAF. Both homogenates were processed simultaneously for biotin switch, followed by streptavidin pull down and Western blotting. All experiments on animals were carried out at the P. Universidad Católica de Chile, approved by the Institutional Bioethics and Biosecurity Committee and conducted according to NIH Guidelines for the use of animals in research.

Identification of S-Nitrosated Cysteines in p120-Catenin

The sites of p120 SNO were identified by MS/MS mass spectrometry. One μ g recombinant p120 was treated with 100 μ mol/L S-nitrosoglutathione 37°C for 30 minutes. The protein was then precipitated 4 times with ice cold acetone (50 μ g lysozyme as a carrier) and washed with ice cold acetone. Free thiols were blocked with 20 mmol/L methyl methanethiosulfonate followed by acetone precipitation and washed with 80% ice cold acetone for 3 times. SNO cysteines were reduced with 10 mmol/L ascorbate and labeled with biotin *N*-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide. The biotinylated sample (about 0.1 μ g p120) was resolved in 8 mol/L urea after acetone precipitation and diluted 10 times using 50 mmol/L NH₄HCO₃ and 0.01 μ g trypsin was added for in solution digestion. The peptides were desalted with a C₁₈ ziptip, and identified by Orbitrap Velos MS. The MS/MS spectra were searched against a Swissprot human database using Thermo proteome Discoverer 1.3.0.339 software with biotin *N*-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide as a variable modification. Peptides identified with 95% confidence interval were displayed in Scaffold3 software. Protein and peptide false discovery rates was <1%.

Statistical Analysis

Experiments were conducted in groups with minimum *n*=3. Data were expressed as mean \pm standard error. Apparent differences were assessed for statistical significance using paired Student *t* test and/or the Newman-Keuls test. Significance was accepted at values of *P*<0.05.

Results

SNO Reduces the Integrity of the Endothelial Barrier

NO derived from eNOS has been largely recognized as a regulatory signal for endothelial permeability; however, the exact mechanisms by which this regulation is achieved are incompletely understood. To investigate how NO modifies proteins of the endothelial barrier to increase microvascular permeability, we applied 10⁻⁷ mol/L PAF to confluent monolayers of bovine coronary venular postcapillary endothelial cells^{21,22} and assessed whether or not PAF-induced release of NO causes SNO of junctional proteins and loss of morphological integrity of the intercellular junctions. We used postcapillary venular endothelial cells initially because these cells are derived from the main site where in vivo regulation of microvascular permeability to macromolecules occurs. We examined the impact of PAF on β -catenin and p120, 2 proteins that are constituents of the adherens junction. Figure 1 shows that in control conditions β -catenin and p120 appear as continuous belt-like structures on the plasma membrane, as revealed by indirect immunofluorescence microscopy. PAF induced significant increment of SNO of β -catenin and p120 as shown by representative Western blot and statistical analysis. PAF-induced SNO was associated with redistribution of these proteins, away from the plasma membrane, and the appearance of a discontinuous pattern on the cell membrane (Figure 1). The increase in SNO was significant at 1 minute after application of PAF, a time lapse that is compatible with a mechanistic causal effect, in accordance with the time course of PAF-induced hyperpermeability.^{2,6,21,23}

SNO Is a Widespread Regulatory Mechanism for the Reorganization of Endothelial Junctional Proteins

To test whether or not the regulation of endothelial permeability to macromolecules by SNO is a widespread mechanism, we tested PAF-induced effects in EAhy926 cells and in ECV-304 cells transfected with a "wild-type" eNOS construct (ECVeNOSGFP), in which eNOS achieves the same distribution found in native endothelial cells.²³ The characteristics of these cells are described in online-only Supplemental Data. In control EAhy926 cells we observed a clear label of β -catenin at the plasma membrane, which tended to disappear with PAF treatment. Likewise the strong fluorescence due to p120 at the plasma membrane observed in control conditions decreased as the result of PAF application (Figure 2A). A similar response pattern was observed in ECVeNOSGFP cells, ie, the immunofluorescence signal for β -catenin and p120 was predominantly at the cell border in control conditions (Figure 2B, arrows) and decreased after 1 minute of PAF treatment. The pattern of both proteins redistribution was maintained at least for 5 minutes during PAF treatment.

The changes in the localization of β -catenin and p120 were correlated with SNO. In control conditions, we detected a basal level of SNO of both proteins in EAhy926 and ECV-eNOSGFP cells (Figure 2A and 2B, lower panels). As

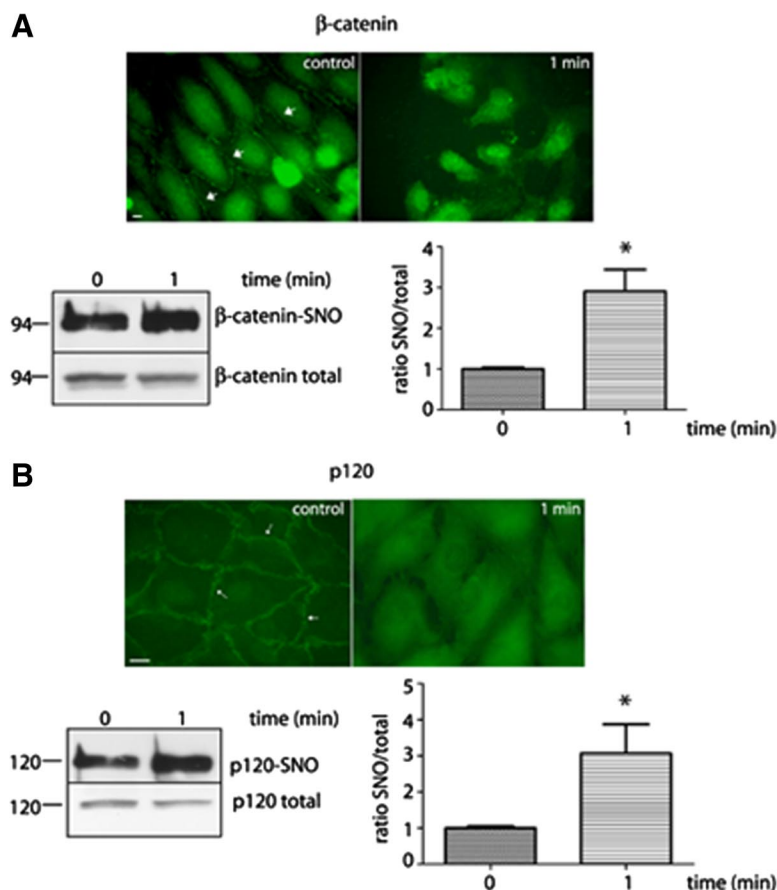


Figure 1. Correlation between protein organization at the cell membrane and S-nitrosation (SNO) of β -catenin and p120 in postcapillary venular endothelial cells. **A, Top:** Indirect immunofluorescence staining for β -catenin in control conditions and after stimulation with 10^{-7} mol/L platelet-activating factor (PAF). **Arrows** indicate the presence of β -catenin in the cell plasma membrane. Bar represents 10 μ m. **Bottom:** SNO of β -catenin as measured by biotin switch. The quantification is shown as the ratio of the S-nitrosylated β -catenin to the total β -catenin on the right lower panel. $*P<0.05$ compared to control; $n=3$. **B, Top:** Indirect immunofluorescence staining for p120 in control conditions and after stimulation with 10^{-7} mol/L PAF. **Left bottom:** SNO of p120 detected by biotin switch. The quantification is shown as the ratio of the S-nitrosylated p120 to the total p120 on the right lower panel. $*P<0.05$ as compared with control; $n=3$.

early as after 1 minute of administration, PAF induced a marked and significant increase in SNO of β -catenin and p120 in both cell models. PAF-induced SNO was associated with reorganization of the distribution of β -catenin and p120 at the plasma membrane in both cell types. Furthermore, these modifications were maintained until the end of the observation period. These observations are consistent with a regulatory association between PAF-induced SNO and PAF-induced hyperpermeability. Interestingly, SNO of β -catenin and p120 was observed also after 15 minutes of continuous application of PAF (Figure 2C).

To confirm that induction of SNO is a common property of proinflammatory agents, we tested whether or not tumor necrosis factor α - α (TNF- α), a well-known proinflammatory agent that increases permeability, recapitulates PAF-induced SNO. Figure 3A shows that TNF- α increased NO production from 8.4 ± 3.7 pmoles/min to 26.9 ± 3.2 pmoles/min at 1 minute after its application. At this time, TNF- α also increased SNO of β -catenin and p120 indicating that SNO is a mechanism shared among agents that cause inflammatory hyperpermeability. To evaluate whether or not release of NO induces nitrosation of junctional proteins independently of changes in permeability, we applied acetylcholine, an agonist that activates eNOS but does not increase permeability.^{23,24} Figure 3B illustrates that acetylcholine does not enhance SNO of β -catenin and p120. In addition, to test whether or not nitrosation-induced hyperpermeability is associated with acti-

vation of sGC, we pretreated the cells with ODQ prior to PAF application. Figure 3C shows that PAF caused a significant increase in permeability to macromolecules in the presence of ODQ, suggesting that PAF-induced SNO of junctional proteins is sufficient to cause hyperpermeability.

eNOS Location Determines SNO of Junctional Proteins

Vasoactive agents induce molecular movement or translocation of eNOS in vitro and in vivo.²⁵ Recently, we demonstrated the importance of localized NO production in hyperpermeability using ECV cells transfected with eNOS constructs that target eNOS to different subcellular locations, GFP-eNOS-G2A (targets to the cytosol) and GFP-eNOS-CAAX (targets to the plasma membrane).^{6,26,27} Even though both cell lines are able to produce NO in response to PAF, only GFP-eNOS-G2A, showing eNOS expression in the cytosol, was able to increase permeability in response to PAF.⁶ On the other hand, ECV-GFP-eNOS-CAAX cells, expressing eNOS at the plasma membrane, did not increase permeability in response to PAF despite producing NO at the same magnitude as ECV-GFP-eNOS-G2A and ECV cells transfected with wild type eNOS. To explore whether or not SNO of junctional proteins depends on eNOS location, we tested PAF-induced SNO of β -catenin and p120 in ECV-GFP-eNOS-G2A and ECV-GFP-eNOS-CAAX cells. Figure 4 displays our results. In agreement with our hypothesis, PAF

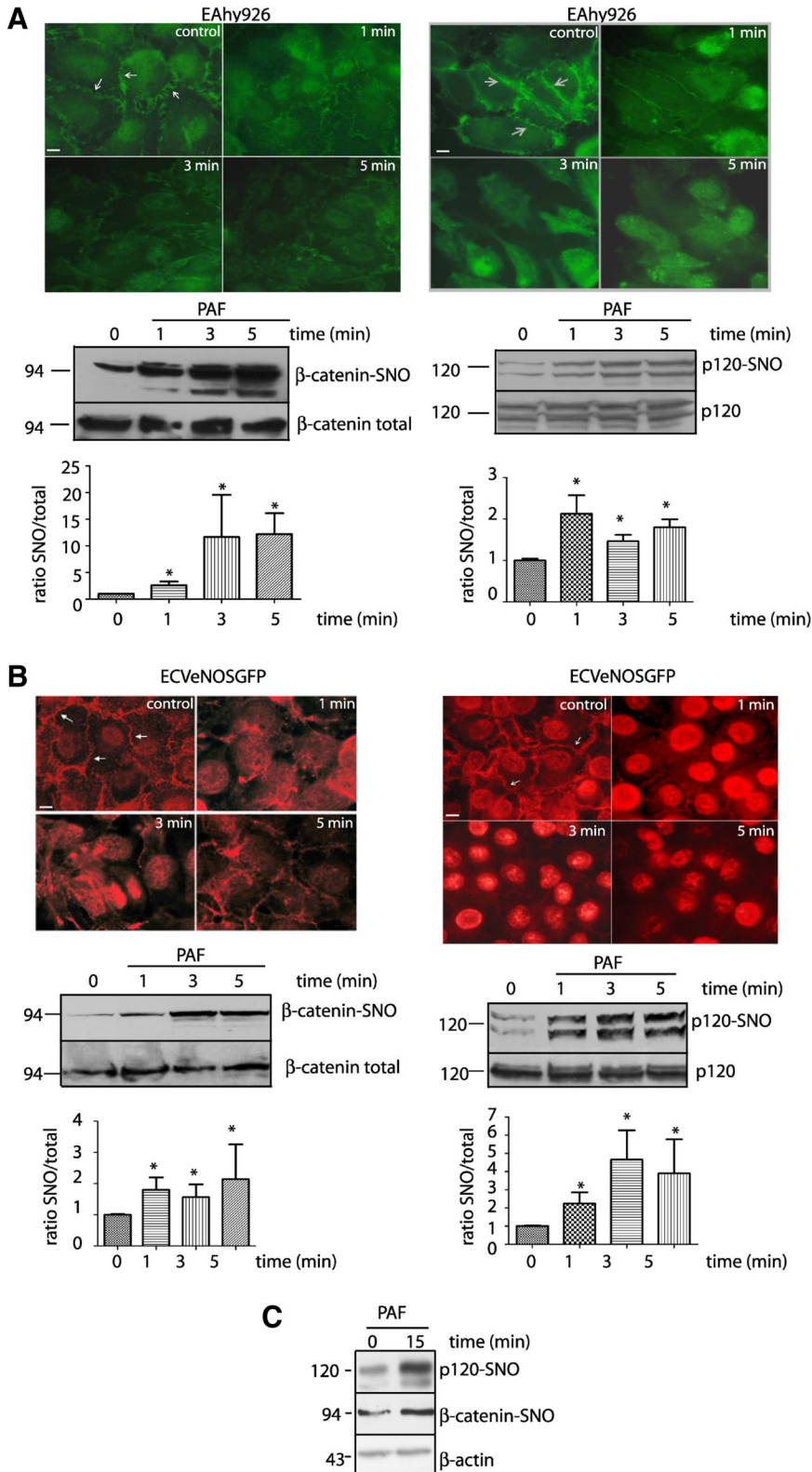


Figure 2. Platelet-activating factor (PAF) induces internalization and S-nitrosation (SNO) of β -catenin and p120 in EAhy926 cells and ECVeNOS-GFP. **A**, Experiments in EAhy926 cells. **Top**: Indirect immunofluorescence staining of EAhy926 cells for β -catenin and p120 after stimulation with 10^{-7} mol/L PAF. **Middle**: SNO of β -catenin (left side) and p120 (right side) as detected by biotin switch. **Bottom**: Bar graph showing the quantification of the SNO signal for β -catenin (left side) and p120 (right side). * $P < 0.05$ compared with control; $n = 3$.

B, Experiments in ECVeNOS-GFP. **Top**: Indirect immunofluorescence staining of ECVeNOS-GFP for β -catenin (left side) and p120 (right side) in control and at different times after stimulation with 5×10^{-7} mol/L PAF. **Middle**: SNO of β -catenin (left side) and p120 (right side) as detected by biotin switch. **Bottom**: Bar graph reporting the quantification of the SNO signal in Western blots for β -catenin (left side) and p120 (right side). * $P < 0.05$ as compared with control; $n = 3$. **C**, Prolonged application of PAF induces SNO of β -catenin and p120 in EAhy926 cells. PAF at 10^{-7} mol/L was applied for 15 minutes. Protein extracts were processed for biotin switch assay and probed with anti- β -catenin and anti-p120 antibodies. Beta-actin was used as a load control. Bars in microphotographs represent 10 μ m.

stimulated SNO of β -catenin and p120 in cells with eNOS targeted to the cytosol (ECV-GFPeNOS-G2A; Figure 4A) but not in cells with eNOS targeted to the plasma membrane (ECV-GFPeNOS-CAAX; Figure 4B). PAF caused significant SNO of junctional proteins only in cells containing eNOS targeted to the cytosol when applied for 1, 3, and 5 minutes,

which correlates well with the PAF-induced reorganization of β -catenin and p120 in adherens junctions in ECV-eNOSGFP (Figure 2). Interestingly, targeting of eNOS to the plasma membrane precluded stimulation of SNO of β -catenin and p120 and is in agreement with our report demonstrating that PAF fails to cause hyperpermeability in these cells.⁶

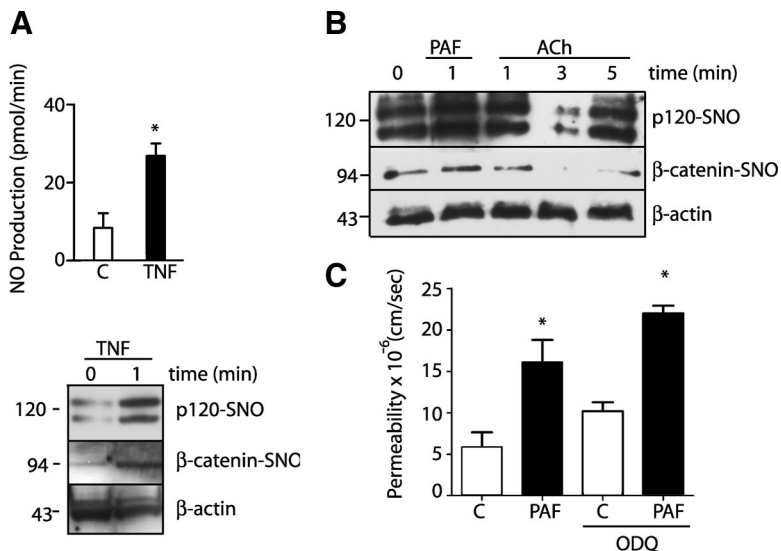


Figure 3. S-nitrosation (SNO) is a widespread regulatory mechanism specific for permeability and independent of sGC and protein kinase G (PKG). **A**, TNF- α induces nitric oxide (NO) production and SNO of β -catenin and p120 in EAh926 cells. Tumor necrosis factor α (TNF- α) at 50 ng/mL was applied for 1 minute. Protein extracts were processed for biotin switch assay and probed with anti β -catenin and anti p120 antibodies. Beta-actin was used as a load control. **B**, ACh at 10^{-5} moles/L does not induce SNO of β -catenin and p120 in EAh926 cells. **C**, PAF at 10^{-7} moles/L increases permeability to FITC-dextran-70 across confluent EAh926 monolayers. PAF-stimulated hyperpermeability is not affected by inhibition of sGC with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). * $P < 0.05$ compared with control; $n = 3$.

SNO Disrupts the Association Between β -Catenin and p120

We investigated whether or not PAF-induced SNO impacts on the association between β -catenin and p120 in ECV-GFPeNOS-G2A and ECV-GFPeNOS-CAAX cells. We tested this concept by immunoprecipitation using an antibody against β -catenin and probing for the presence of p120 in the precipitate. The data were quantified as a ratio to the total content of β -catenin in the input and compared to the levels seen at time 0. Figure 5 demonstrates that PAF significantly decreased the association between β -catenin and p120 in ECV-GFPeNOS-G2A cells (cytosolic eNOS cells) when applied for 1, 3, and 5 minutes (Figure 5A). Interestingly, PAF failed to alter the association between β -catenin and p120 in ECV-GFPeNOS-CAAX cells (plasma membrane eNOS; Figure 5B). These data confirm the importance of

eNOS location in regards to the ability of PAF to elicit NO-dependent functional reactions and support the concept that increases in permeability are related to modifications in the association between proteins that make up the junctional complex.

SNO of Junctional Proteins Increases Permeability to Macromolecules

To assess directly the cause-effect relationship between SNO and hyperpermeability to macromolecules, we measured PAF-induced transport of FITC-dextran 70 (molecular weight: 70 kDa) across confluent monolayers of EAh926 cells under control conditions as well as under inhibition of eNOS by L-NMA (L-N-methyl arginine) and inhibition of SNO by NAC (N-acetyl-L-cysteine). Figure 6A shows that

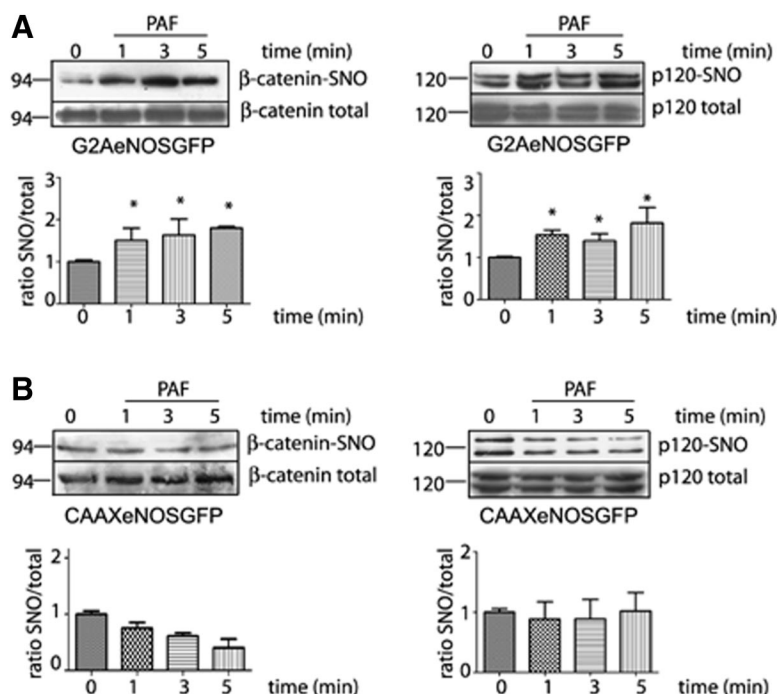


Figure 4. Subcellular location of endothelial nitric oxide synthase (eNOS) determines S-nitrosation (SNO) of β -catenin and p120. Protein extracts from ECV-GFPeNOS-G2A and ECV-GFPeNOS-CAAX cells treated with 5×10^{-7} mol/L platelet-activating factor (PAF) for different times were processed for biotin switch assay and probed with anti β -catenin and anti p120 antibodies. **A, Top**: PAF-induced SNO of β -catenin and p120 in ECV-GFPeNOS-G2A cells. **Bottom**: Bar graph showing the quantification of the Western blots of β -catenin (left side) and p120 (right side). * $P < 0.05$ compared to control; $n = 3$. **B, Top**: PAF-induced SNO of β -catenin and p120 in ECV-GFPeNOS-CAAX cells. **Bottom**: Bar graph showing the quantification of the Western blots of β -catenin (left side) and p120 (right side). * $P < 0.05$ compared to control; $n = 3$.

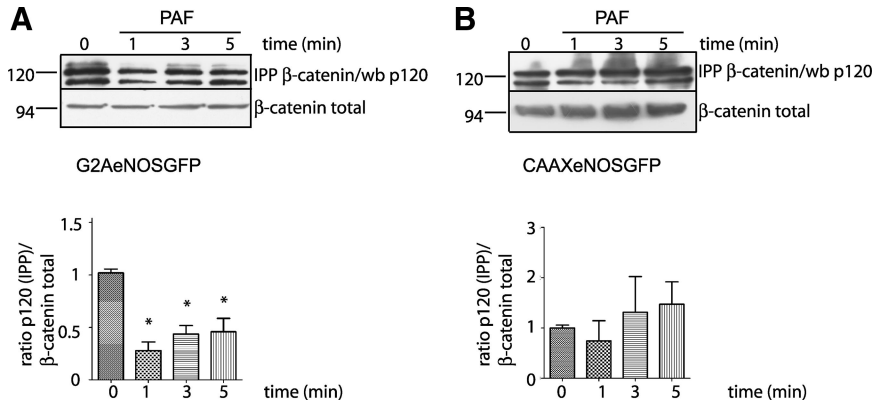


Figure 5. S-nitrosation (SNO) disrupts the association between β -catenin and p120. Protein extracts from ECV-GFPeNOS-G2A and ECV-GFPeNOS-CAAX cells, treated with 5×10^{-7} mol/L platelet-activating factor (PAF) for different times, were immunoprecipitated (IPP) with anti β -catenin antibodies and probed by Western blot with anti-p120 antibodies. **A**, Experiments in ECV-GFPeNOS-G2A cells. A representative Western blot assessing the association between β -catenin and p120 in ECV-GFPeNOS-G2A cells treated with PAF for different times is shown on the upper section, with corresponding statistical analysis illustrated below. Time indicates the duration of PAF application. $*P < 0.05$ as compared with control; $n = 3$. **B**, Experiments in ECV-GFPeNOS-CAAX cells. The upper section shows a representative Western blot assessing whether or not PAF stimulates the disruption of the association between β -catenin and p120 in ECV-GFPeNOS-CAAX cells. The statistical evaluation is illustrated in the lower section. No statistical significance was observed.

both NAC and L-NMA significantly inhibit PAF-induced hyperpermeability to FITC-dextran 70. The abolition of hyperpermeability was associated with an effective reduction of PAF-induced SNO of β -catenin and p120 in strong support of a cause-effect relationship between PAF-induced SNO and PAF-induced hyperpermeability to macromolecules (Figure

6B). Pretreatment of the cells for 15 minutes with 500 μ mol/L N-ethylmaleimide (which blocks sulfhydryl-groups impairing NO binding) strongly blocks SNO of β -catenin and p120 in response to PAF (Figure 6C). In further support of our hypothesis, we showed that NAC and L-NMA prevented the PAF-induced disruption of the association

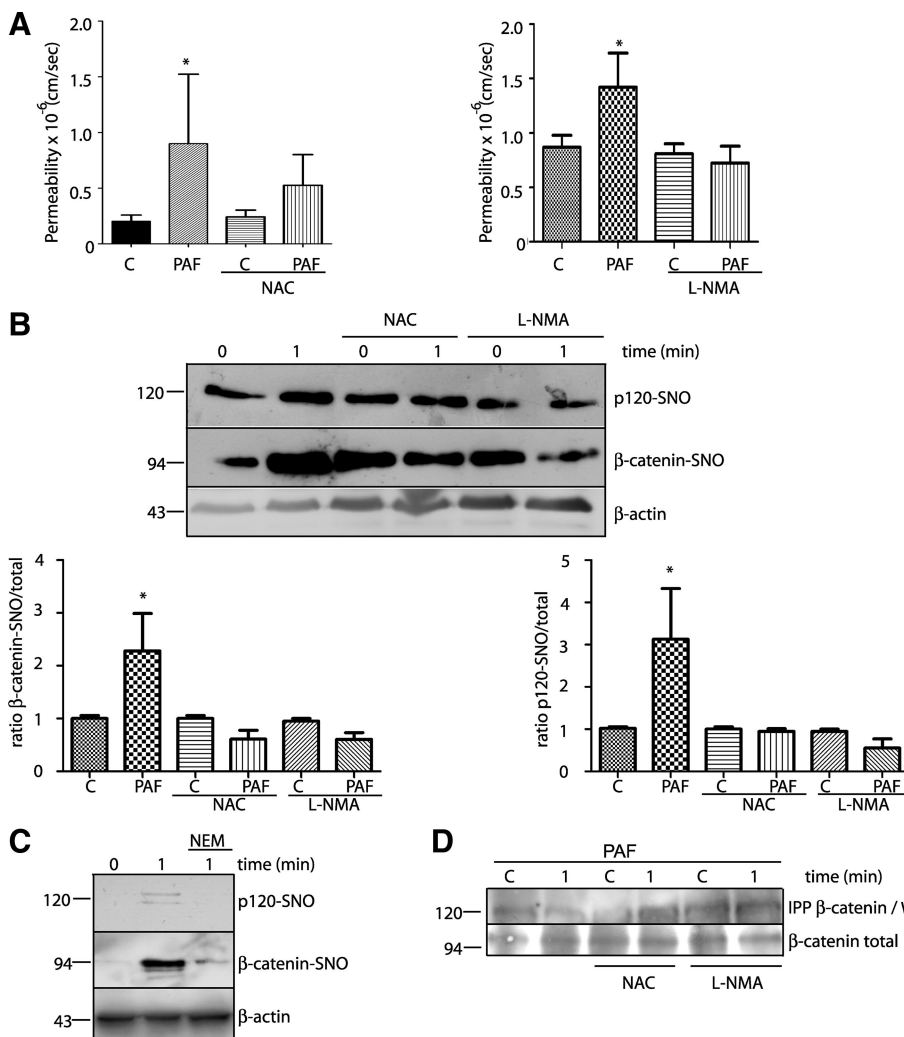


Figure 6. Platelet-activating factor (PAF)-induced hyperpermeability to macromolecules correlates strongly with S-nitrosation (SNO) of β -catenin and p120 in EAhy926 cells. **A**, PAF-induced hyperpermeability in EAhy926 cells is blocked by inhibition of SNO with N-acetyl-L-cysteine (NAC; **left side**) and by inhibition of endothelial nitric oxide synthase (eNOS) with NG-methyl-L-arginine (L-NMA; **right side**). C=control. Data are expressed as mean permeability \pm SEM. $*P < 0.05$ relative to control; $n = 3$. **B**, PAF-induced SNO of β -catenin and p120 is prevented by NAC, a competitive inhibitor of SNO, and by L-NMA, an inhibitor of eNOS. $*P < 0.05$ as compared with control; $n = 3$. **C**, PAF-induced SNO of β -catenin and p120 is prevented by N-ethylmaleimide (NEM), a blocker of sulfhydryl groups. **D**, Protein extracts from EAhy926 cells treated with 10^{-7} moles/L PAF for 1 minute, in the presence of NAC or L-NMA, were immunoprecipitated for β -catenin and probed by Western blot with anti-p120 antibodies. The representative blot shows that NAC and L-NMA blocked PAF-induced disruption of the association between β -catenin and p120.

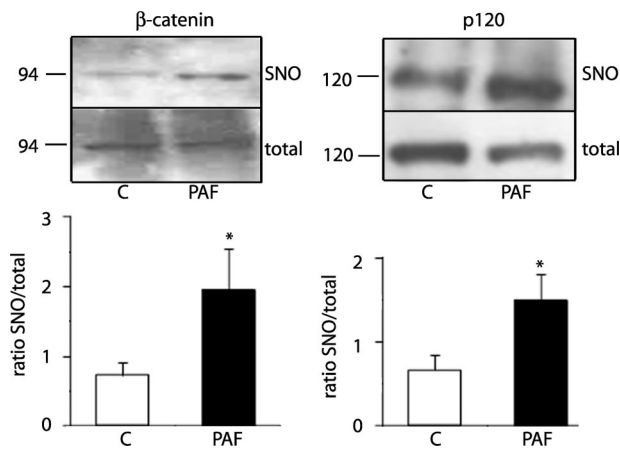


Figure 7. Platelet-activating factor (PAF) induces S-nitrosation (SNO) of β -catenin and p120 in the in vivo mouse cremaster muscle. PAF, at 10^{-7} moles/L, was applied to 1 cremaster muscle for 3 minutes while buffer was applied to the other cremaster (control) in the same animal. Homogenized tissues were prepared for biotin switch assay and streptavidin pull down to determine β -catenin and p120 SNO. The **top** panels show representative Western blot. The **lower** panels display the ratio of SNO to total protein (input) for each junctional protein. * $P < 0.05$ as compared with control; $n = 6$.

between β -catenin and p120 (Figure 6C). Taken together, these data demonstrate SNO-mediated disruption of the interaction between β -catenin and p120 at the adherens junction is a relevant mechanism by which eNOS-derived NO regulates endothelial permeability.

PAF Induces SNO In Vivo

To assess whether or not SNO is a mechanism that causes hyperpermeability in vivo, we administered 10^{-7} mol/L PAF to the mouse cremaster muscle and evaluated nitrosation by the biotin switch assay. PAF application resulted in significant SNO of β -catenin and p120 (Figure 7). The data, obtained at 3 minutes of continuous PAF application, are in agreement with previous reports demonstrating eNOS-derived NO induced hyperpermeability.¹

Identification of S-Nitrosated Cysteines in p120

To determine the cysteines nitrosated by NO in p120, we exposed $1 \mu\text{g}$ of purified p120 to $100 \mu\text{mol/L}$ S-nitrosoglutathione at 37°C

for 30 minutes (see Supplemental Data for a detailed account). Analysis by mass spectroscopy identified 5 cysteines that can be S-nitrosated: C429, C450, C579, C618, and C692 (Figure 8). Of these amino acids, C579 is the only residue that was S-nitrosated in 100% of the assayed peptides. C579 locates at helix 3 of repeat 5 of the Armadillo domain, a region that is important for the interactions of p120 with E-cadherin.^{42,43,28} Therefore, the modification of this cysteine could affect the interaction with VE-cadherin.

Discussion

Our results advance the novel concept that location of eNOS is important for effective SNO of endothelial junctional proteins by NO, in particular β -catenin and p120. Our data provide strong support for the hypothesis that SNO of junctional proteins leads to hyperpermeability. Importantly, we show that SNO of β -catenin and p120 is a powerful mechanism that contributes to PAF-stimulated hyperpermeability in vivo.

Increases in microvascular permeability to macromolecules via paracellular pathways have been attributed to 2 main mechanisms: a) cytoskeletal contraction mediated by myosin light chain²⁹ and b) phosphorylation of adherens junction proteins that causes their internalization away from the adherens junctions.^{30,12} These processes lead to reorganization of the adherens junctional complex and allow transport of macromolecules across endothelial monolayers. The process of permeability regulation is more complex as modifications of cyclic nucleotides by phosphodiesterases and changes in eNOS activity (leading to nitrosation and/or nitration) may play a role in reducing and enhancing the barrier properties of the endothelium.^{32,33} Although nitric oxide has been reported as an agent that contributes to decrease microvascular permeability³⁴ as well as to increase it,^{3,35} the importance of NO in promoting the onset of endothelial permeability in response to proinflammatory agents has been unequivocally demonstrated using eNOS knockout mouse^{1,36} and cells treated with siRNA for eNOS.²

There are no reports of direct effects of NO in the adherens junction in response to PAF in the literature. Using 3 different cell models (postcapillary venular endothelial cells, EAhy926 and ECV-eNOSGFP cells), we demonstrate SNO of

| | | | | | | |
|-----|------------|------------|------------|------------|------------|------------|
| 1 | MDDSEVESTA | SILASVKEQE | AQFEKLTRAL | EEERRHVSQA | LERVRVSPQD | ANPLMANGTL |
| 61 | TRRHQNGRFV | GDADLERQKF | SDLKLNQPD | HSLLYSTIP | RMQEPGQIVE | TYTEEDPEGA |
| 121 | MSVSVETSD | DGTRRTTETT | VKKVVKTVT | RTVQPVAMGP | DGLPVDASSV | SNNYIQLTGR |
| 181 | DFRKNNGGGP | GPYVQGAGTA | TLPRNFHYPP | DGYSRHYEDG | YPGSDNYGS | LSRVTRIEER |
| 241 | YRPSMEGYRA | PSRQDVYGPQ | PQVRVGGSSV | DLHRFHPEPY | GLEDDQSRMG | YDDLQYGMMS |
| 301 | DYGTARTGT | PSDPRRLRS | YEDMIGEEVP | SDQYYWAPLA | QHERGSLASL | DSLKKGPPFP |
| 361 | PNWRQPELPE | VIAMLGFRLL | AVKSNAAYL | QHLCYRNDKV | KTDVRLKGI | PVLVGLDHP |
| 421 | KKEVHLGAG | ALKNISFGRD | QDNKIAIKNC | DGVPAVLRL | RKARDMLTE | VITGTLWNLS |
| 481 | SHDSIKMEIV | DHALHALTDE | VIIPHSGWER | EPNEDCKPRH | IEWESVLTNT | AGCLRNVSE |
| 541 | RSEARRKLRE | CDGLVDALIF | IVQAEIGQKD | SDSKLVENCV | CLLRNLSYQV | HREIPQAERY |
| 601 | QEAAPNVANN | TGPHAAAFG | AKKGKDEWFS | RGKKPIEDPA | NDTVDFPKRT | SPARGYELLF |
| 661 | QPEVVRIYIS | LLKESKTPAI | LEASAGAIQN | LCAGRWYGR | YIRSALRQEK | ALSAIADLLT |
| 721 | NEHERVVKAA | SGALRNLAVD | ARNKELIGKH | AIPNLVKNL | GGQNSWNF | SEDTVISILN |
| 781 | TINEVIAENL | EAAKKLRETQ | GIEKLVLINK | SGNRSEKEVR | AAALVLQTIW | GKELRKPLE |
| 841 | KEGWKKSDFQ | VNLNNASRSQ | SSHSDYDSTL | PLIDRNQKSD | KKPDREEIQM | SNMGSNTKSL |
| 901 | DNNYSTPNER | GDHNRTLDRS | GDLGDMEPLK | GTTPLMQDEG | QESLEELD | LVLDEGGQV |
| 961 | SYPSMQKI | | | | | |

Figure 8. Mapping of S-nitrosation sites in p120. Protein sequence from human p120. Purified p120 was S-nitrosated with S-nitrosoglutathione for 30 minutes, subjected to biotin switch assay followed by in-solution trypsin digest. The region that potentially interacts with vascular endothelial-cadherin is shown in gray background. Cysteine 579 (white letter, red background) was 100% S-nitrosated according to mass spectrometry. Other cysteines (red background) were not S-nitrosated in 100% of the assays. Unlabeled cysteines were not S-nitrosated. (See online-only Supplemental Data for experimental details.)

β -catenin and p120 in response to PAF. This modification leads to changes in the localization and interaction between these proteins at the adherens junction, which finally leads to hyperpermeability. This is the first report linking NO signaling directly to p120 as a target at the adherens junction. Furthermore, we demonstrate that this mechanism does occur in vivo as PAF caused SNO of β -catenin and p120 in the mouse cremaster muscle at times corresponding to the onset of the hyperpermeability response.

The classic dogma in NO signaling establishes that the actions of NO are mediated via sGC and PKG. However, increases in permeability occur in some cellular models regardless of PKG activation but still require NO production.¹⁴ Our results after inhibition of sGC with ODQ agree with the findings that synthesis of cGMP and activation of PKG are not essential for the development of hyperpermeability in response to PAF (Figure 3). Recently, SNO has emerged as an important NO-dependent posttranslational modification (independent of sGC/PKG pathway) of free-thiol cysteines that alters the function of proteins, affecting processes of intracellular trafficking and phosphorylation and requires proximity between eNOS and the target proteins for appropriate NO delivery.^{15,16,17} Our results showing SNO of β -catenin and p120 in cells with cytosolic eNOS strongly confirm the requirement of proximity to induce the modification. The fact that plasma membrane-anchored eNOS fails to induce SNO of these proteins despite that it efficiently produces NO strongly supports the concept that SNO depends on NO local concentration and the relevance of directed SNO as a mechanism to increase permeability. In support of these concepts, we demonstrate that acetylcholine—an agonist that localizes eNOS to Golgi, stimulates production of NO and induces vasodilation^{24,37,23}—does not cause SNO. PAF may cause enhanced permeability by formation of peroxynitrite,³⁸ the reaction product of NO and superoxide anion.³⁹ In the hamster cheek pouch, 3-morpholinysydnonimine N-ethylcarbamide,⁴⁰ a peroxynitrite generator, induces hyperpermeability.⁴¹ However, we did not pursue this line of inquiry because peroxynitrite preferentially leads to protein nitration;⁴² ie, protein modification via NO binding to tyrosine rather than by protein nitrosation of cysteines.

Our combined data make a compelling case for SNO of key junctional proteins induced by eNOS-derived NO as a novel mechanism for regulation of permeability to macromolecules across endothelial cell monolayers and the microvasculature. Dissociation between β -catenin and p120 from VE-cadherin disrupts the junctional complex and leads to loss of barrier integrity and enhanced transport of molecules across endothelial monolayers and/or postcapillary venules.³¹ We focused on β -catenin and p120 as relevant targets. The interactions of β -catenin and p120 with VE-cadherin are required for the barrier function. In fact, the absence of p120⁴³ and β -catenin^{44,45,43} causes loss of barrier integrity and an increase in permeability even in the presence of VE-cadherin. Recently, SNO of β -catenin was associated with changes in permeability in response to 15-minute application of vascular endothelial growth factor.⁴⁶ While confirming SNO of β -catenin, we demonstrate the novel observation

that SNO occurs at early times that are consistent with the onset of hyperpermeability in response to proinflammatory agonists. In addition, we provide novel evidence showing that SNO of p120 is an important molecular mechanism in PAF-induced hyperpermeability. The relationships and interactions between SNO and phosphorylation, an established regulatory mechanism, remain to be elucidated. Recent evidence suggests that SNO plays an important role because mutation of C619, which is subject to SNO, was sufficient to inhibit Vascular endothelial growth factor-induced hyperpermeability even though phosphorylation increased in response to the agonist.⁴¹ In the case of p120 the phosphorylation sites are in the regulatory domain and tail domain, whereas the cysteines that are available for SNO are in the region of possible interactions between p120–VE-cadherin. From these observations, it is possible to speculate that parallel synergism may exist between phosphorylation and SNO in the process of internalization of junctional proteins.

Based on our mass spectrometry results, we propose that C579 is a strong candidate for SNO-regulation by NO in vivo because it is located in the molecular stretch that interacts with E-cadherin.^{47,48} Because of the high homology between E-cadherin and VE-cadherin, it is plausible that SNO of this cysteine affects the interactions of p120 with VE-cadherin. Modification of cysteines by NO is a new chemical mechanism that alters protein-protein interactions at the cell junction and causes enhanced permeability.

Our data demonstrating SNO of β -catenin and p120 in response to PAF and TNF- α confirm and expand the observation that eNOS-derived NO causes nitrosation of β -catenin in response to VEGF.⁴⁶ These combined data suggest that SNO of junctional proteins may be a universal mechanism shared by proinflammatory agonists. Importantly, we advance the field by demonstrating that SNO of junctional proteins occurs at times compatible with the onset of hyperpermeability. The NO modification of the cysteines is a new mechanism that alters protein-protein interactions at the cell junction and causes enhanced permeability.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Nitric oxide is a key factor in regulating microvascular permeability.
- Platelet-activating factor (PAF) increases permeability via translocation of endothelial nitric oxide synthase to cytosol.
- Increases in permeability can be stimulated independently of soluble guanylyl cyclase-protein kinase G activation.

What New Information Does This Article Contribute?

- S-nitrosation of p120-catenin and β -catenin increases endothelial permeability to macromolecules.
- Increase in permeability in response to PAF can occur independently of soluble guanylyl cyclase-protein kinase G activation.
- PAF induced S-nitrosation of junctional proteins β -catenin and p120 and their internalization away from the plasma membrane.
- Endothelial nitric oxide synthase located in the cytosol, but not plasma membrane-anchored endothelial nitric oxide synthase, causes S-nitrosation of β -catenin and p120.
- S-nitrosation of β -catenin and p120 significantly diminished association between these proteins.
- Mass spectrometry analysis of purified p120 identified cysteine 579 as the main S-nitrosated residue.

During acute inflammation many mediators act on endothelial cells increasing microvascular permeability. Endothelial adherens junctions constitute an important element in the control of microvascular permeability. The mechanisms by which nitric oxide signaling regulates permeability at adherens junctions are still incompletely understood. We demonstrate S-nitrosation of β -catenin and p120 in response to PAF. This modification leads to changes in the localization and interaction between these proteins at the adherens junction, which leads to hyperpermeability. This is the first report linking nitric oxide signaling directly to p120 as a target at the adherens junction. Furthermore, we demonstrate that this mechanism does occur *in vivo* as PAF caused S-nitrosation of β -catenin and p120 in the mouse cremaster muscle at times corresponding to the onset of the increase in permeability. We identify cysteine 579 in p120 as the main residue S-nitrosated during the increase in permeability. We anticipate that our results demonstrating the significance of S-nitrosation in regulation of microvascular permeability may serve as a basis for the development of new therapeutic strategies in the treatment of vascular diseases characterized by inflammation.