BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Site-Specific S-Nitrosylation of Integrin α6 Increases the Extent of Prostate Cancer Cell Migration by Enhancing Integrin β1 Association and Weakening Adherence to Laminin-1

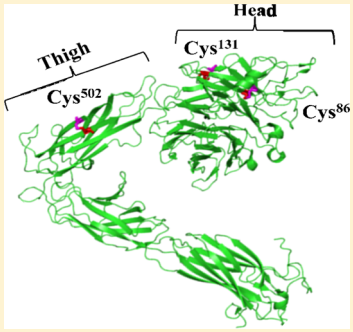
Jared Isaac,[†,‡] Pheruza Tarapore,[‡,§,∥] Xiang Zhang,[‡,§,∥] Ying-Wai Lam,[‡] and Shuk-Mei Ho*[,‡,§,∥,⊥]

[†]Cancer and Cell Biology, [‡]Department of Environmental Health, [§]Center for Environmental Genetics, and [∥]Cancer Institute, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267, United States

[⊥]Cincinnati Veterans Affairs Hospital Medical Center, Cincinnati, Ohio 45220, United States

Supporting Information

ABSTRACT: The increased mortality in prostate cancer is usually the result of metastatic progression of the disease from the organ-confined location. Among the major events in this progression cascade are enhanced cell migration and loss of adhesion. Moreover, elevated levels of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) found within the tumor microenvironment are hallmarks of progression of this cancer. To understand the role of nitrosative stress in prostate cancer progression, we investigated the effects of NO and iNOS on prostate cancer cell migration and adhesion. Our results indicate that ectopic expression of iNOS in prostate cancer cells increased the extent of cell migration, which could be blocked by selective ITGα6 blocking antibody or iNOS inhibitors. Furthermore, iNOS was found to cause S-nitrosylation of ITGα6 at Cys86 in prostate cancer cells. By comparing the activities of wild-type ITGα6 and a Cys86 mutant, we showed that treatment of prostate cancer cells with NO increased the level of ITGα6 heterodimerization with ITGβ1 but not with ITGβ4. Finally, S-nitrosylation of ITGα6 weakened its binding to laminin-β1 and weakened the adhesion of prostate cancer cells to laminin-1. In conclusion, S-nitrosylation of ITGα6 increased the extent of prostate cancer cell migration, which could be a potential mechanism of NO- and iNOS-induced enhancement of prostate cancer metastasis.



Prostate cancer (PCa) is the second most common cancer in American men[1] and will kill ∼28000 patients in 2012 primarily because of metastatic disease.[2] To reduce the mortality from this cancer, it is therefore imperative to understand how PCa cells escape the primary tumor and spread to secondary sites. A loss of cellular adhesion and an increase in cell motility are major events in this metastatic cascade.

Nitric oxide (NO), a free radical gas, has been shown to play an important role in tumor progression.[3,4] It is synthesized from L-arginine by NO synthases (NOSs).[5] Three major human isoforms have been identified, namely, neuronal, endothelial, and inducible (nNOS, eNOS, and iNOS, respectively).[6] Endothelial and neuronal NOSs are constitutively expressed and responsible for maintaining low levels (nanomolar range) of NO production in a cell-type-specific manner. In contrast, iNOS produces a large output (micromolar range) of NO in response to inflammatory cytokines or pathogens as part of the host defense mechanism.[6,7] At low levels, NO is a ubiquitous signaling molecule that regulates normal cellular functions,[5,7] while chronic high levels of NO contribute to the development of various diseases, including PCa.[8−10]

In PCa, NO promotes tumor initiation and progression.[9,10] Inducible NOS was found to be overexpressed in high-grade prostatic intraepithelial neoplasia (HGPIN)[9] and adenocarcinoma,[10] as well as in their surrounding inflammatory cells when compared with levels expressed in adjacent nonmalignant tissue.[11] It has been postulated that the overexpression of iNOS promotes PCa cell growth and survival, DNA damage, angiogenesis, invasiveness, and metastasis during the development and progression of the cancer through an increased level of NO production,[9−12] yet the downstream effectors and the mode of action of NO and iNOS in prostate carcinogenesis remain to be identified.

An important means by which NO regulates cellular functions is through post-translational modification of signaling proteins at their cysteine residues via a process called S-nitrosylation.[13] Site-specific S-nitrosylation alters the function, stability, subcellular localization, and binding partners of its target proteins.[14] Inappropriate S-nitrosylation of key regulatory proteins disrupts normal physiological function and leads to the pathogenesis of diseases.[15−17]

Integrins are expressed in all epithelial cells and have diverse functions in regulating cell morphology, cell−cell interaction, and signal transduction from the extracellular matrix.[18] An altered expression or aberrant distribution of integrins disrupts the cell−substratum relationship, increases cell motility, and promotes the progression of epithelial cancers, including PCa.[19]

Received: September 10, 2012
Revised: October 16, 2012
Published: October 29, 2012

dx.doi.org/10.1021/bi3012324 | *Biochemistry* 2012, 51, 9689−9697

Using the biotin switch technique (BST),[20] we recently conducted a site-specific mapping of the S-nitrosoproteome in an immortalized prostate epithelial cell line, NPrEC,[21] and identified integrin $\alpha 6$ (ITG$\alpha 6$) as a target for S-nitrosylation at cysteines 86, 131, and 502. BST substitutes biotin for the labile and otherwise difficult to detect NO moiety, making identification of S-nitrosylation more readily achievable.[32]

In normal prostate epithelial cells, ITG$\alpha 6$ binds specifically to integrin $\beta 1$ (ITG$\beta 1$) or ITG$\beta 4$ to form $\alpha 6\beta 4$ or $\alpha 6\beta 1$ integrin. These are receptors of prostate acinar laminins, allowing cells to adhere to the basement membrane.[19,22] However, during PCa progression, while multiple integrins, including ITG$\beta 4$, are downregulated, both ITG$\alpha 6$ and ITG$\beta 1$, subunits of $\alpha 6\beta 1$, are overexpressed in PCa cells and in their corresponding lymph node metastases,[23,24] suggesting that ITG$\alpha 6$ and/or ITG$\beta 1$ expression favors PCa cell metastasis.[25] This report is the first to address the question of whether S-nitrosylation plays a role in regulating the function of ITG$\alpha 6$ in PCa progression. Here, we report that (i) ectopic expression of iNOS or treatment with a NO donor stimulates PCa cell migration through ITG$\alpha 6$, (ii) iNOS associates with ITG$\alpha 6$ and induces S-nitrosylation of the integrin, and (iii) substitution of Cys86 with serine, when compared to substitution at the other two sites, most significantly affected the iNOS- and NO-induced S-nitrosylation of ITG$\alpha 6$ in PCa cells. We further demonstrate that S-nitrosylation of ITG$\alpha 6$ Cys86 enhanced binding of the integrin subunit to ITG$\beta 1$, but not to ITG$\beta 4$, and weakened its adhesion to laminin-$\beta 1$. These data suggest site-specific S-nitrosylation of ITG$\alpha 6$ is part of the mechanism underlying the iNOS- and NO-mediated promotion of PCa cell migration.

EXPERIMENTAL PROCEDURES

Cell Culture. All cells were maintained in a humidified incubator at 37 °C with 5% CO_2 as previously described.[26]

Vector Production. Plasmids expressing ITG$\alpha 6$ (BC136455) and iNOS (BC130283) (Thermo Fisher, Boston, MA) were cloned into the pcDNA3.1/TOPO expression vector according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). ITG$\alpha 6$ was C-terminally tagged with the Flag epitope (DYKDDDDK). Mutagenesis of cysteines 86 (TGC → TCC), 131 (TGT → TCT), and 502 (TGT → TCT) to serines (C86S, C131S, and C502S, respectively) was accomplished via Stratagene QuikChange XL site-directed mutagenesis (Agilent, Santa Clara, CA) of pcDNA3.1/ITG$\alpha 6$-Flag constructs. Ectopic expression of iNOS or a control vector (LacZ) in PC-3 cells was accomplished using lentivirus constructs as previously described.[27]

Cell Transfection and Infection. HEK-293 cells were transfected using Lipofectamine 2000 (Invitrogen), and PC-3 cells were transfected using the Mirus TransIT-Prostate Transfection Kit (MirusBio Corp., Madison, WI) according to the manufacturer's protocol for 24 h unless otherwise noted. PC-3 and DU-145 cells were infected as previously described.[27]

NO Donor Preparation, Nitrite Measurement, and the Biotin Switch Technique (BST). *S*-Nitroso-L-cysteine (CysNO) and *S*-nitrosoglutathione (GSNO) were freshly prepared as previously described.[21] CysNO and GSNO were used for experiments with NO donors needed for short and long time points, respectively. The Griess–Saltzman assay was used to detect the level of nitrite in cell culture media according to the manufacturer's protocol (Invitrogen) in triplicate. The BST[20] was performed as previously described.[21] All experiments were performed in triplicate.

Gel Electrophoresis and Western Blot Analysis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed with 8% gels under nonreducing conditions in triplicate. After electrophoresis, gels were transferred onto a PVDF membrane (Immobilon FL, Millipore, Billerica, MA) with a blotting cell (Invitrogen). The following antibodies were used: Flag (2368, Cell Signaling, Danvers, MA), β-actin (A2228, Sigma-Aldrich, St. Louis, MO), ITG$\alpha 6$ [SC-6597, Santa Cruz Biotechnology (SCBT), Santa Cruz, CA], ITG$\beta 4$ (SC-6629, SCBT), iNOS (SC-651, SCBT), laminin-$\beta 1$ (SC-17810, SCBT), ITG$\beta 1$ (MAB1987Z, Millipore), and rabbit IgG (SC-2027, SCBT). The ITG$\alpha 6$ antibody (GoH3) blocks the interaction of ITG$\alpha 6$ with laminin and was a generous gift from A. Sonnenberg.[28,29] For all inhibition studies, 10 μg/mL GoH3 was used. Primary antibodies were used at 1:1000 dilutions overnight at 4 °C or for 1 h followed by the corresponding IRDye-conjugated secondary antibodies (LICOR Biosciences, Lincoln, NE) used at 1:15000 dilutions as previously described.[21]

Wound Healing. Cells were allowed to reach confluence, serum-starved for 24 h, and wounded as previously described[30] in triplicate. Specifically, cells were treated with a NO donor and/or ITG$\alpha 6$ inhibiting antibody, GoH3, at the indicated doses. Pictures were taken at 0, 8, and 24 h using a Zeiss microscope (Carl Zeiss Microimaging, GmbH) at 40× magnification. Migratory distances were measured using Zeiss Axiovision. Wells were coated overnight with 10 μg/mL laminin-1 and fibronectin (Sigma Aldrich) at 4 °C. For the antibody inhibition experiments, all antibodies were used at a concentration of 10 μg/mL. Experiments were performed in triplicate.

Boyden Chamber Assay. Cell invasion was measured using the Boyden chamber assay (BD Biosciences, Raleigh, NC) as previously described[30] with the following modifications. Invasion chambers were coated with 10 μg/mL human laminin-1 (Sigma Aldrich) overnight at room temperature followed by blocking with a 0.1% BSA/PBS mixture prior to seeding cells.

Cell Viability. We measured cell viability by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) reagent, diluting the cells (1:5) in cell medium, and incubating them for 1 h after the experiment. Viable cells were identified colorimetrically by setting the absorbance at 490 nm directly proportional to the number of living cells in culture.

BrDU Incorporation. DNA synthesis was assessed according to the manufacturer's protocol (Millipore).

Cell Adherence. Cell adhesion experiments were performed as previously described[29] in triplicate. Specifically, wells were coated with 10 μg/mL human laminin-1 (Sigma Aldrich) followed by blocking with a 0.5% BSA/PBS mixture. Wells were washed with a 0.1% BSA/PBS mixture before 40000 cells were seeded. Cells were allowed to adhere for 1 h at 37 °C. Nonadherent cells were aspirated, and wells were washed four times via rotation at 70 rpm. Adherent cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Plates were washed followed by methanol incubation. Adherent cells were stained with crystal violet and read spectrophotometrically at 490 nm. Cell adherence was calculated as the ratio of cells that adhered to laminin-1 to those that adhered to polylysine. Experiments were performed in triplicate.

Co-Immunoprecipitation. Lysates were co-immunoprecipitated according to a published protocol.[31] Specifically, cells were seeded on plates coated with 10 μg/mL laminin-1 and lysed with radio-immunoprecipitation assay (RIPA) buffer (Sigma Aldrich) with protease inhibitors. Lysates were incubated with 2 μg/mL primary or control antibodies for 16 h at 4 °C. The primary antibodies were pulled down with protein A/G Plus agarose beads (SC-651 SCBT). Laminin-β1 was chosen for laminin-1 co-immunoprecipitation because it is known to interact with ITGα6.[28] Experiments were performed in triplicate.

Statistical Analyses. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). ImageJ for quantification of band intensity on a Western blot was downloaded from http://rsbweb.nih.gov/ij/index.html.

RESULTS

NO and iNOS Stimulate PCa Cell Migration via ITGα6 Action. To determine whether S-nitrosylation plays a role in regulating the function of ITGα6 in PCa progression, we tested whether increased levels of NO and iNOS enhance PCa cell migration and invasion, key events in cancer progression. To do this, we first determined the concentration of nitrosoglutathione (GSNO) tolerated by PC-3 cells by measuring the effect of increasing GSNO concentrations on PC-3 cell viability (Figure 1A). Because the 100 μM GSNO dose was tolerated well, we tested its effect upon cell invasion using Boyden chambers coated with laminin-1. In the presence of the 100 μM GSNO dose, there was a significant increase in the extent of cell invasion (Figure 1B). Cell invasion is thought to result from changes in two cellular functions: (i) remodeling of the cellular adhesion interactions both with adjoining cells and with the extracellular matrix and (ii) cell migration. To confirm the effects of GSNO on cell migration, we used the in vitro wound healing assay. Moreover, because high levels of NO in the prostate tumor microenvironment had been attributed to high levels of iNOS in HGPIN,[9] adenocarcinoma,[10] and invading inflammatory cells,[11] we either treated the two PCa cell lines, PC-3 and DU-145, with GSNO or transfected them with an iNOS expression plasmid (Figure 1F). Twenty-four hours after the cells had been wounded, a significant increase in the extent of migration was observed in these cell lines after treatment with GSNO, when compared with their respective untreated controls (Figure 1C). Additionally, iNOS expression resulted in a statistically significant, increased level of cell migration compared to the empty vector controls (Figure 1C, bottom panels, EV and iNOS). To confirm that ectopic expression of iNOS increased the level of NO production, the levels of nitrite were measured in cell culture media at the end of the wound healing assay (Figure 1A,B of the Supporting Information). A significant increase in the levels of measurable nitrite was observed in PCa cell cultures 24 h after transfection with the iNOS expression plasmid (Figure 1C of the Supporting Information) even with a transfection rate of 40−60% (not shown). The nitrite levels in the culture media from these cells were comparable to those obtained from cells treated with 100 μM GSNO. Furthermore, elevated levels of NO persisted for at least 48 h after either treatment (Figure 1C of the Supporting Information). A weakness of the wound healing assay is that the observed cell migration could be a result of cell proliferation. We hence checked that the observed increase in the extent of cell migration was not an artifact of an enhancement of cell proliferation. Neither GSNO nor ectopic iNOS expression

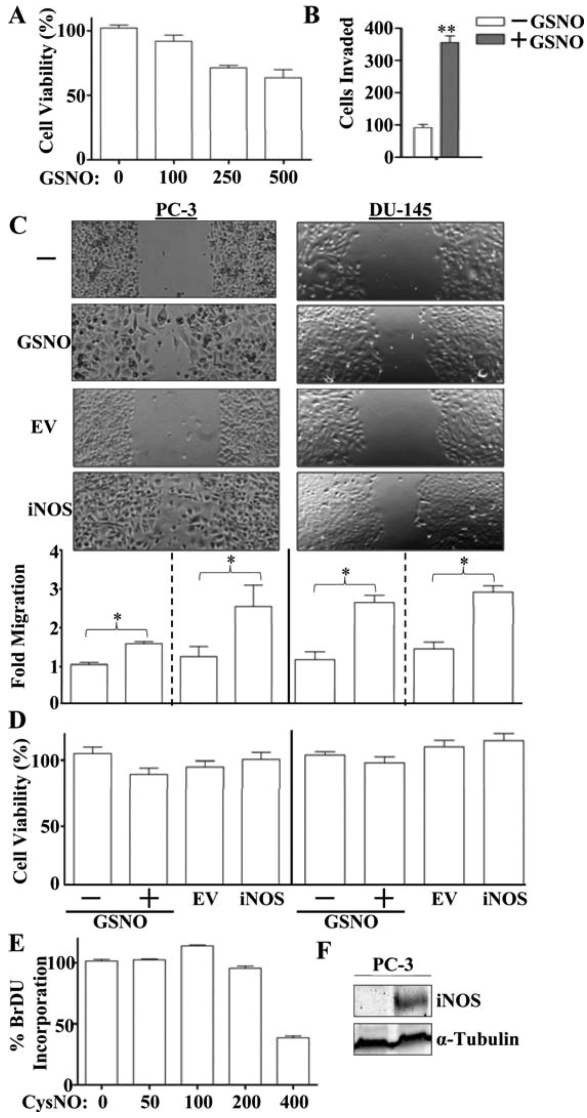


Figure 1. iNOS increases the extent of migration of prostate cancer cells. (A) PC-3 cells were treated with a range of GSNO concentrations (0, 100, 250, and 500 μM) for 24 h. (B) PC-3 cells treated with or without 100 μM GSNO for 24 h were subjected to the invasion assay. Asterisks denote $p < 0.01$. (C) PC-3 (left) and DU-145 (right) cells were subjected to the wound healing assay with or without 100 μM GSNO, empty vector (EV), or iNOS expressing vector. (D) PC-3 and DU-145 cells were treated with or without 100 μM GSNO and transfected with EV or iNOS for 24 h followed by the MTS cell viability assay (Promega). (E) PC-3 cells were treated with a range of CysNO concentrations (0, 50, 100, 200, and 400 μM) for 24 h followed by analysis of the BrDU DNA synthesis assay (Millipore). (F) Western blot showing iNOS transfection efficiency (MirusBio Corp.) after transfection for 24 h, with 100 μg loaded on an 8% gel. All experiments were conducted three times, except for the invasion assay (twice).

altered cell viability in the treated cultures (Figure 1D). Using a range of nitrocysteine concentrations, DNA synthesis was only significantly altered at higher concentrations and not at a 100 μM CysNO dose (Figure 1E).

To demonstrate enhanced expression of iNOS is causally linked to an increased extent of cell migration, the wound healing assay was performed in PC-3 cultures transfected with a control or iNOS-expressing plasmid in the presence or absence of an iNOS inhibitor, 1400W. We found that iNOS-mediated

dx.doi.org/10.1021/bi3012324 | Biochemistry 2012, 51, 9689−9697

enhancement of cell migration in PC-3 cells 8 h after they had been wounded was ameliorated upon treatment with 1400W (Figure 2A). To test the hypothesis that NO induces PCa cell migration through integrin action per se, we performed the wound healing assay with PC-3 and DU-145 cultures treated with GSNO in wells coated with two different extracellular matrices, laminin-1 and fibronectin. GSNO increased the extent of PC-3 and DU-145 cell migration with laminin-1 but not fibronectin (Figure 2B). To determine which integrin was influencing prostate cancer cell migration with laminin-1 in the presence of GSNO, the wound healing assay was performed in the presence or absence of antibodies targeted against integrins αV, α6, β1, and β4. Integrin α6 and β1 antibodies were the only ones that affected cell migration of PC-3 cells (Figure 2C, white bars). Moreover, the antibodies against integrins α6 and β1 were found to block the GSNO-mediated enhancement of cell migration in these cultures (Figure 2C). The increase in the extent of migration seen in the presence of GSNO is not statistically significant for integrin α6 and β1 antibodies (Figure 2C, gray bars).

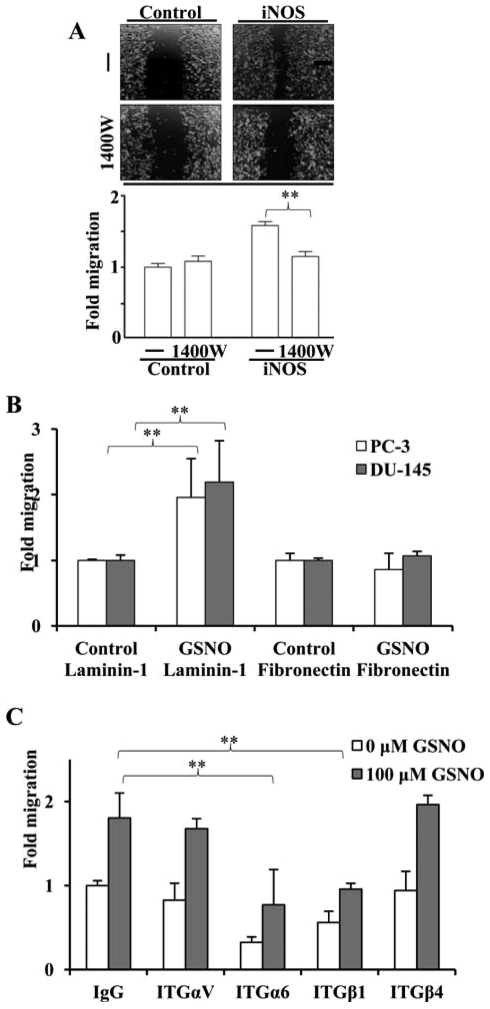


Figure 2. iNOS- and NO-mediated cell migration is blocked by 1400W or GoH3. (A) PC-3 cultures infected with LacZ (control) or an iNOS expression plasmid were wounded in the absence or presence of 100 μM 1400W for 8 h. (B) PC-3 and DU-145 cells were wounded upon laminin-1-coated or fibronectin-coated (10 μg/mL, each) plates in the absence or presence (control or GSNO) of 100 μM GSNO for 8 h. (C) PC-3 cells were wounded, treated in the absence (white bars) or presence (gray bars) of 100 μM GSNO, and incubated with anti-ITGαV, -ITGα6, -ITGβ1, and -ITGβ4 antibodies for 8 h. IgG was used as an isotype control. All experiments were conducted three times. Asterisks denote $p < 0.01$.

ITGα6 Interacts with iNOS and Is S-Nitrosylated by Increased Levels of NO or iNOS Expression. Co-immunoprecipitation experiments were conducted to examine whether iNOS associates with ITGα6. Cell lysates from PC-3 cells infected with the iNOS expression virus or LacZ expression virus were immunoprecipitated with anti-iNOS, anti-ITGα6, or IgG control antibodies and subjected to Western blot analysis. ITGα6 was found to co-immunoprecipitate with iNOS (Figure 3A, lane 3), suggesting the possibility of an association between these two proteins. To determine whether iNOS S-nitrosylates ITGα6, HEK-293 cells transiently transfected with a control or ITGα6-Flag expression plasmid were subjected to the BST in the presence or absence of the NO donor CysNO or following the transfection of an iNOS expression plasmid. Western blot analyses revealed that S-nitrosylation of ITGα6 occurred in the presence of CysNO or ectopic expression of iNOS (Figure 3B, lanes 3 and 4), but not in its absence (lane 2). To demonstrate endogenous ITGα6 can be S-nitrosylated in PCa cells, PC-3 cells were infected with the iNOS expression virus or treated with CysNO and subjected to the BST (Figure 3C). S-Nitrosylated ITGα6 was detected only in PC-3 cells treated with the NO donor or ectopically expressing iNOS (Figure 3C, lanes 2 and 3), not in control PC-3 cells (lane 1). ITGα6 was also S-nitrosylated in LNCaP and DU-145 cells (Figure 2 of the Supporting Information).

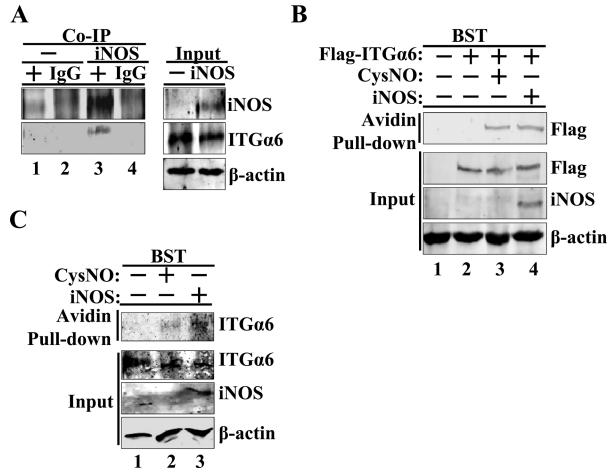


Figure 3. ITGα6 interacts with iNOS and is S-nitrosylated with increased levels of NO or iNOS expression in cellulo. (A) iNOS was co-immunoprecipitated (Co-IP) with ITGα6 using from PC-3 lysates infected with LacZ (−) or the iNOS expression plasmid. A plus sign indicates iNOS antibody immunoprecipitation. (B) The BST was conducted using lysates from HEK-293 cells transfected with or without an ITGα6-Flag plasmid following treatment with 1 mM CysNO or transfected with an iNOS expression plasmid (iNOS). (C) The BST was conducted in PC-3 cells with or without treatment with 1 mM CysNO or transfection of an iNOS expression plasmid. All experiments were conducted three times.

Cys86 Is Central in ITGα6 S-Nitrosylation. We previously reported the treatment of NPrEC cells with CysNO-induced S-nitrosylation of 172 proteins.[21] ITGα6 was identified to be in this S-nitrosoproteome and the S-nitrosylation shown to occur at Cys86, Cys131, and Cys502

dx.doi.org/10.1021/bi3012324 | Biochemistry 2012, 51, 9689−9697

by mass spectrometry.[21] As indicated in the cartoon of ITGα6, these sites are located in the extracellular head (Cys86 and -131) and thigh (Cys502) regions of ITGα6 (Figure 4A). To

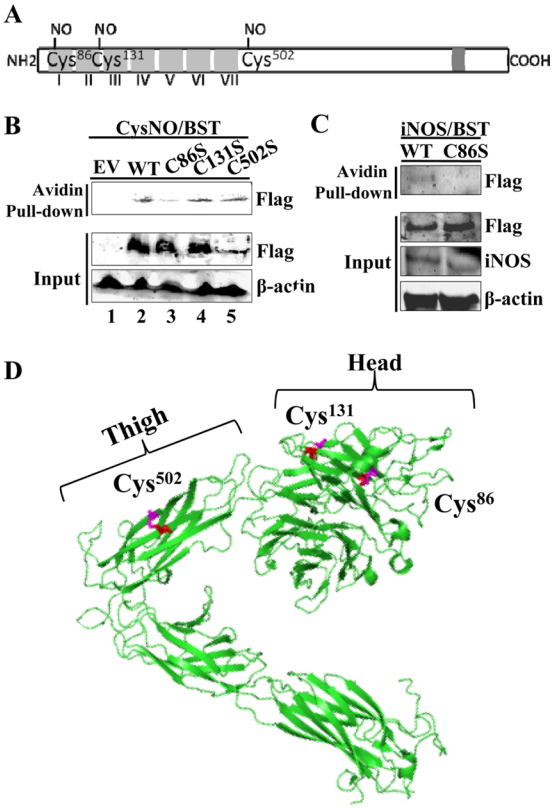


Figure 4. iNOS S-nitrosylates ITGα6 at Cys86. (A) Cartoon model of ITGα6 indicating S-nitrosylation sites relative to homologous repeat domains I–VII colored gray and the transmembrane domain colored dark gray. (B) PC-3 cells stably expressing ITGα6-Flag with cysteines 86, 131, and 502 mutated to serines were treated with 1 mM CysNO and subjected to the BST. (C) WT/PC-3 and PC-3/C86S cells were transfected with the iNOS expression plasmid and subjected to the BST. (D) ITGα6 was modeled using the ITGαV crystal structure (Protein Data Bank entry 3IJE) to visualize S-nitrosylated Cys86, -131, and -502 (red) with their predicted cysteine binding partners (magenta) within the "head" and "thigh" tertiary domains of ITGα6. All experiments were conducted three times.

determine which of these three cysteines is the most important site mediating ITGα6 S-nitrosylation, PC-3 cells stably expressing the empty vector (PC-3/EV), wild-type ITGα6-Flag (PC-3/WT), or ITGα6-Flag with a C86S (PC-3/C86S), C131S (PC-3/C131S), or C502S (PC-3/C502S) substitution were generated. These cell lines were treated with CysNO and subjected to BST analysis (Figure 4B, top panel). We observed a marked decrease in the level of S-nitrosylated ITGα6 (Flag-pull down) in PC-3/C86S cells (Figure 4C, lane 3) but not in PC-3/C131S or PC-3/C502S cells (Figure 4C, lanes 4 and 5). These results indicate that biochemically, Cys86 is the most important site mediating S-nitrosylation of ITGα6. To demonstrate the physiological significance of Cys86 in S-nitrosylation of ITGα6, PC-3/WT and PC-3/C86S cells were transfected with the iNOS expression plasmid and subjected to BST analysis (Figure 4C). No S-nitrosylated ITGα6/C86S protein was pulled down by avidin in the PC-3/C86S cell line, whereas ITGα6 protein was readily detected in PC-3/WT cells, suggesting that the Cys86 residue is important for ITGα6 S-nitrosylation by iNOS.

We next conducted the wound healing assay with PC-3/WT and PC-3/C86S cells with or without treatment with GSNO, in the presence and absence of the anti-ITGα6 GoH3 antibody, which blocks the interaction of integrin with laminin.[28] The antibody was found to block the NO-induced enhancement of cell migration in PC-3/WT cells, but not in PC-3/C86S cells (Figure 3A of the Supporting Information).

To visualize how site-specific S-nitrosylation may affect the function of ITGα6, we modeled the tertiary structure of the protein, including the three S-nitrosylation sites (Figure 4D), using the available crystal structure of ITGαV that has a primary sequence significantly similar to that of ITGα6. The S-nitrosylated cysteine residues Cys86, Cys131, and Cys502 are conserved in ITGαV, and their positions are colored red with their predicted cysteine binding partners colored magenta in Figure 4D. While Cys86 and Cys131 are found to be located within the head region of ITGα6, Cys502 is located within the thigh region. On the basis of structural analysis, the Cys86–Cys95 loop located within the head region is largely exposed to the solvent, suggesting that Cys86 could be transiently exposed on the surface of ITGα6. Approximate normal-mode analysis performed using Elastic Network Model, as implemented in the NOMAD-ref server (http://lorentz.immstr.pasteur.fr/nomad-ref.php), lends further support to the idea that the Cys86 residue contributes significantly to slow coordinated motions in ITGα6 (data not shown). Taken together, experimental and modeling results indicate that S-nitrosylation of ITGα6 at Cys86 may be significantly functionally relevant. Hence, subsequent functional studies were performed using PC-3/WT and PC-3/C86S cells.

ITGα6 Cys86 Is Required for the NO-Induced Increase in the Extent of PC-3 Cell Migration and Enhanced Association with ITGβ1. To determine the consequence of a C86S substitution with respect to the NO-induced enhancement of cell migration, wound healing assays were performed with ITGα6 PC-3/WT and PC-3/C86S cells treated with GSNO or the vehicle control (Figure 5A). As shown previously (Figure 1A), NO significantly increased the extent of migration of PC-3/WT cells but not that of PC-3/C86S cells (Figure 5A).

We then examined the effects of NO on the association of ITGα6 with ITGβ1 or ITGβ4 and determined whether the process involves Cys86. PC-3/WT and PC-3/C86S cells were seeded onto laminin-1-coated plates and treated with GSNO for 8 h. Lysates obtained from PC-3/WT cells had more ITGα6-associated ITGβ1 following GSNO treatment compared to levels in lysates from vehicle-treated controls (Figure 5B, lane 1 vs lane 3, top panel, and histograms). This difference cannot be completely ascribed to the NO-induced increase in the level of ITGα6 expression (lane 1 vs lane 3, bottom panel). In contrast, GSNO treatment did not affect the amount of ITGβ4 associated with ITGα6 (lane 1 vs lane 3, middle panel). Importantly, the amount of ITGα6 and the degree of association of ITGβ1 with ITGα6 in PC-3/C86S cells were insensitive to GSNO treatment (lane 5 vs lane 7, top, middle, and bottom panels).

To determine the effect of the Cys86 substitution, we performed the invasion assay with ITGα6 PC-3/WT and PC-3/C86S cells treated with GSNO or the vehicle control. After 24 h, there was a significant increase in the extent of invasion for PC-3/WT cells but not for PC-3/C86S cells (Figure 5C).

dx.doi.org/10.1021/bi3012324 | Biochemistry 2012, 51, 9689–9697

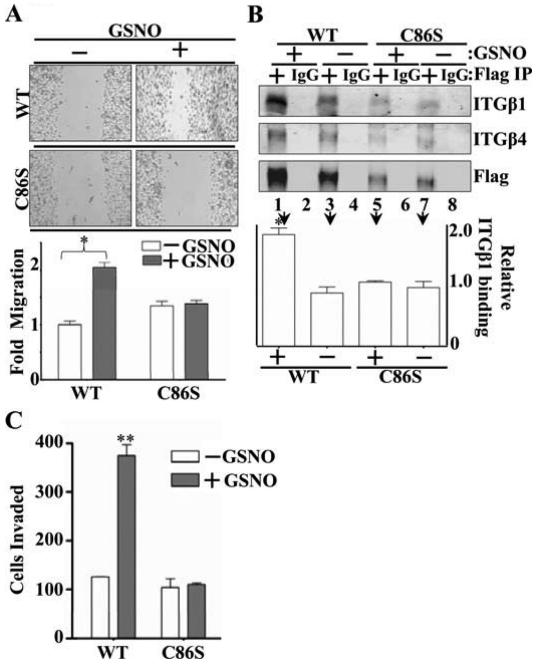


Figure 5. NO mediates migration through ITGα6 Cys86 and promotes enhanced ITGβ1 heterodimerization. (A) PC-3/WT and PC-3/C86S cells were wounded for 8 h with or without 100 μM GSNO. The asterisk denotes $p < 0.05$. (B) PC-3/WT and PC-3/C86S cells were seeded onto plates precoated with 10 μg/mL laminin-1 and allowed to adhere for 8 h with or without 100 μM GSNO. ITGα6 was immunoprecipitated using a Flag antibody, and its associated ITGβ1 or ITGβ4 was detected by Western blotting. Quantitation was conducted with ImageJ by measuring ITGβ1 bands and subtracting them from WT and C86S Flag immunoprecipitation bands. WT Flag/ITGβ1 without NO bands was set to the baseline. (C) PC-3/WT and PC-3/C86S cells were seeded onto Boyden chambers coated with 10 μg/mL laminin-1 and allowed to invade for 8 h with or without 100 μM GSNO. Asterisks denote $p < 0.01$. All experiments were conducted three times, except for the invasion assay (twice).

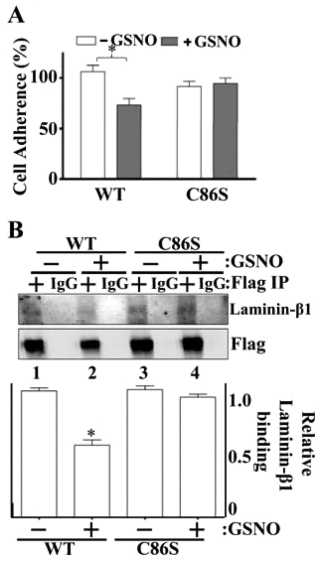


Figure 6. Cys86 is responsible for NO destabilization of ITGα6-mediated cell adherence. (A) WT/PC-3 and C86S cells were seeded onto plates precoated with human laminin-1 or polylysine and allowed to adhere for 1 h in the presence or absence of 500 μM GSNO. The percent cell adherence was calculated as the cell absorbance on laminin-1 divided by the absorbance on polylysine × 100%, with values observed in untreated WT/PC-3 cells arbitrarily set to 100%. The asterisk denotes $p < 0.05$. (B) WT/PCS and C86S/PC-3 cells that adhered to laminin-1 for 1 h in the presence 500 μM GSNO were used to prepare cell lysates for immunoprecipitation with the Flag antibody. Laminin-β1 was chosen for laminin-1 co-immunoprecipitation because it is known to mediate interactions with ITGα6. Quantitation of WT and C86S Flag IP bands relative to their laminin-β1 bands was done with ImageJ. All experiments were conducted three times.

S-Nitrosylation of ITGα6 at Cys86 mediates the NO-induced reduction in the level of adherence of cells to laminin-1. We examined the effect of NO on ITGα6-mediated cell adhesion to laminin-1, which is composed of α1, β1, and γ1 subunits; its other name is laminin-111. PC-3/WT and PC-3/C86S cells were seeded on laminin-1-coated plates in the presence or absence of GSNO for 1 h. Treatment with GSNO caused a significant decrease in the level of adherence of PC-3/WT cells but exerted no effect on the adherence of PC-3/C86S cells to laminin-β1 (Figure 6A). GSNO treatment did not cause a significant alteration of adhesion of PC-3 cells to polylysine alone (Figure 3B of the Supporting Information). In concordance, while treatment of PC-3/WT cells with GSNO weakened ITGα6−laminin-1 interaction (Figure 6B, lane 1 vs lane 2), it has no effect on the PC-3/C86S cell line (lane 3 vs lane 4), as demonstrated in experiments using Flag-ITGα6 immunoprecipitation followed by Western blotting for laminin-β1. These results indicate that the disruption of cell adhesion after GSNO treatment is accompanied by a reduction in the extent of ITGα6−laminin-β1 interaction, with both processes likely mediated by S-nitrosylation of ITGα6 at Cys86.

DISCUSSION

Integrins are important mediators of cell adhesion and migration.[18] In this study, we showed that NO and iNOS significantly increased the extent of PCa cell migration and decreased the extent of cell adhesion through S-nitrosylation of Cys86 on ITGα6. This post-translational modification was further shown to promote the interaction of ITGα6 with ITGβ1, but not ITGβ4, and to weaken its association with laminin-β1. These findings are consistent with reports demonstrating a positive association between iNOS expression and PCa progression and metastasis in clinical specimens[9−11] and the pivotal role of perturbations of cancer cell−matrix interaction in PCa progression.[32]

Our findings provide the first evidence that S-nitrosylation of an integrin (ITGα6) promotes PCa cell migration in a site-specific manner. Although earlier studies have reported NO promotes cell migration in a variety of cells such as eosinophils[33] and mammary cancer cells,[34] few have definitively demonstrated S-nitrosylation of motility-related molecules at specific cysteines as a causative factor. This initial lack of progress was in part due to the very unstable nature of the S-nitrosylated proteins. With the development of the BST, the highly unstable protein SNOs are replaced with biotin that can be readily detected as well as sequenced for the identification of the S-nitrosylation sites.[20,35] Using the BST, S-nitrosylation of actin in neutrophils was shown to alter actin polymerization, network formation, intracellular distribution, and interaction with integrins.[36] Similarly, both NO and estradiol-17β activate c-Src through S-nitrosylation of its Cys498 in MCF-7 cells and promote cancer cell invasion and metastasis.[37]

Our data suggest iNOS may directly interact with ITGα6 and mediate S-nitrosylation of the integrin. In fact, overexpression

dx.doi.org/10.1021/bi3012324 | Biochemistry 2012, 51, 9689−9697

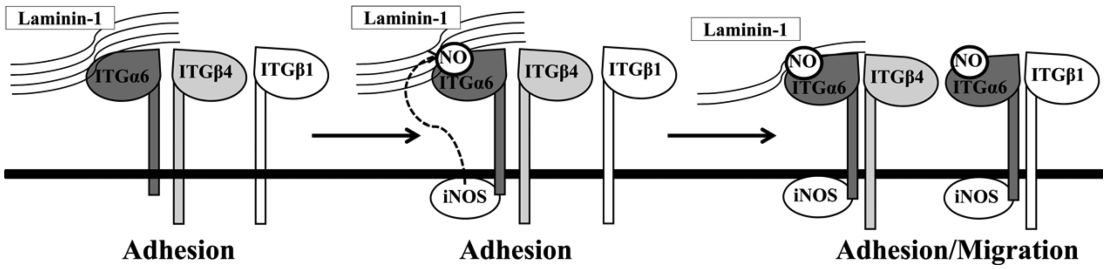


Figure 7. Proposed model of how iNOS may affect binding of ITGα6 to laminin-1 and heterodimerization to ITGβ4 and ITGβ1, resulting in weakened adherence and enhanced cell migration.

of iNOS is more effective than GSNO presumably because iNOS can directly interact with ITGα6 and therefore directly S-nitrosylate ITGα6. This postulate is consistent with the general belief that the selectivity of S-nitrosylation is provided by protein−protein interaction like that of the S-nitrosylation of cyclooxygenase-2[38] and caspase-3.[39] However, because Cys86, identified as being essential for S-nitrosylation, is located in the head region that normally resides outside the cell membrane, it raises the question of how intracellular iNOS can S-nitrosylate this site. One probable mechanism is the individual association of iNOS[40] and integrins[41] with caveolin-1, bringing these two molecules to the caveolae, the plasma membrane organelle responsible for recycling integrins and transducing matrix information during cell migration.[42] Whether iNOS-induced S-nitrosylation of ITGα6 occurs within the caveolae remains to be established. Alternatively, it is possible that the NO released by intracellular iNOS diffuses across the cell membrane and S-nitrosylates ITGα6 on the outside of the cell.

From modeling ITGα6, we recognize that Cys86 lies at the bottom of an easily exposed loop and, in contrast, Cys131 is fully buried. Therefore, the S-nitrosylation of Cys86 might lead to exposure of Cys131 and its subsequent S-nitrosylation, so in the absence of Cys86 S-nitrosylation, Cys131 may not be accessible for S-nitrosylation. In this regard, Cys86 may be the first in a cascade of S-nitrosylation of a series of cysteines, including Cys131 and Cys502, analogous to the sequential phosphorylation of multiple sites at the cytoplasmic tails of receptor tyrosine kinases.[43] Thus, in silico predictions could explain the experimental observation that Cys86 is the most sensitive S-nitrosylation site on ITGα6. However, future investigations are needed to ascertain whether sequential or cooperative S-nitrosylation of multiple cysteines in ITGα6 contributes to the regulation of the action of this integrin.

S-Nitrosylation of Cys86 on ITGα6 was found to be essential and sufficient to mediate the NO- and iNOS-induced enhancement of PCa cell migration and loss of matrix adhesion. These results are consistent with findings from a series of studies reporting that NO regulates the disulfide bonds in extracellular proteins, including integrins.[44−46] S-Nitrosylation of specific cysteines in integrins induces breakage, re-formation, or reshuffling of disulfide bonds, resulting in conformational changes in these molecules.[47] These changes ultimately lead to functional changes, including alterations in ligand binding and matrix adhesion, and cell motility for these integrins.[48,49] Such structural and functional changes probably occur upon S-nitrosylation of Cys86 on ITGα6, resulting in a decreased extent of cell adhesion and an increased extent of cell migration.

At the molecular level, we showed that S-nitrosylation of Cys86 favors the association of ITGα6 with ITGβ1 (and not ITGβ4) and weakens its binding to laminin-1 and laminin-β1. We suggest that NO strengthens its interaction with ITGβ1, and we showed that ITGβ4 heterodimerization remains the same. These findings are in agreement with those of previous studies[22] reporting increased levels of production and formation of integrin $\alpha6\beta1$ and laminin as promoters of tumor progression and metastasis that is commonly accompanied by a loss of $\alpha6\beta4$. One probable mechanism underlying the S-nitrosylation-induced alterations in heterodimer preference and matrix protein affinity could be the result of breaking and/or reshuffling of disulfide bonds at targeted cysteines as discussed above. Another likely mechanism may involve the cleavage of ITGα6 at Arg594 and -595 that has previously been shown to play a significant role in PCa migration and metastasis.[50] However, although Cys502 is in the proximity of Arg594 and -595, it remains to be determined if S-nitrosylation of this site mediates or facilitates ITGα6 cleavage.

In conclusion, iNOS induced an increased extent of migration of PCa cells via S-nitrosylation of ITGα6 at Cys86 that is likely the trigger of enhanced ITGα6−ITGβ1 heterodimerization and loss of binding of ITGα6 to laminin-β1. These biochemical and molecular changes were accompanied by weakened cell adherence and increased motility in PCa cells. A schematic model has been proposed (Figure 7). Given these observations, inhibiting S-nitrosylation has potential for use in prostate cancer therapy and prevention.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental Figures 1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Environmental Health, Kettering Complex, Room 128, 3223 Eden Ave., University of Cincinnati Medical Center, P.O. Box 670056, Cincinnati, OH 45267-0056. Telephone: (513) 558-5701. Fax: (513) 558-0071. E-mail: shuk-mei.ho@uc.edu.

Funding

This work was supported in part by National Institutes of Health Grants ES006096, EB012122, ES006096, ES019480, and ES020988 (S.M.H.), Veteran Affairs Grant BX000675 (S.M.H.), Congressionally Directed Medical Research Program Department of Defense Grant PC10835 (J.I.), and Grant PC094619 (P.T.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Thanks to Arnoud Sonnenberg from the Department Cell Biology of The Netherlands Cancer Institute (Amsterdam, The Netherlands), who graciously provided the GoH3 antibody, and Jarek Meller from the Division of Biomedical Informatics, University of Cincinnati, for the modeling studies of ITGα6.

■ ABBREVIATIONS

NO, nitric oxide; iNOS, inducible nitric oxide synthase; ITGα6, integrin α6; ITGβ4, integrin β4; ITGβ1, integrin β1; BST, biotin switch technique.

■ REFERENCES

(1) Jemal, A., Siegel, R., Xu, J., and Ward, E. (2010) Cancer statistics, 2010. *Ca-Cancer J. Clin. 60*, 277−300.

(2) Siegel, R., Naishadham, D., and Jemal, A. (2012) Cancer statistics, 2012. *Ca-Cancer J. Clin. 62*, 10−29.

(3) Lala, P. K., and Chakraborty, C. (2001) Role of nitric oxide in carcinogenesis and tumour progression. *Lancet Oncol. 2*, 149−156.

(4) Thomsen, L. L., and Miles, D. W. (1998) Role of nitric oxide in tumour progression: Lessons from human tumours. *Cancer Metastasis Rev. 17*, 107−118.

(5) Moncada, S., and Higgs, A. (1993) The L-arginine-nitric oxide pathway. *N. Engl. J. Med. 329*, 2002−2012.

(6) Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001) Nitric oxide synthases: Structure, function and inhibition. *Biochem. J. 357*, 593−615.

(7) Martinez-Ruiz, A., Cadenas, S., and Lamas, S. (2011) Nitric oxide signaling: Classical, less classical, and nonclassical mechanisms. *Free Radical Biol. Med. 51*, 17−29.

(8) Xu, W., Liu, L. Z., Loizidou, M., Ahmed, M., and Charles, I. G. (2002) The role of nitric oxide in cancer. *Cell Res. 12*, 311−320.

(9) Baltaci, S., Orhan, D., Gogus, C., Turkolmez, K., Tulunay, O., and Gogus, O. (2001) Inducible nitric oxide synthase expression in benign prostatic hyperplasia, low- and high-grade prostatic intraepithelial neoplasia and prostatic carcinoma. *BJU Int. 88*, 100−103.

(10) Aaltoma, S. H., Lipponen, P. K., and Kosma, V. M. (2001) Inducible nitric oxide synthase (iNOS) expression and its prognostic value in prostate cancer. *Anticancer Res. 21*, 3101−3106.

(11) Uotila, P., Valve, E., Martikainen, P., Nevalainen, M., Nurmi, M., and Harkonen, P. (2001) Increased expression of cyclooxygenase-2 and nitric oxide synthase-2 in human prostate cancer. *Urol. Res. 29*, 23−28.

(12) Vanella, L., Di, G. C., Acquaviva, R., Santangelo, R., Cardile, V., Barbagallo, I., Abraham, N. G., and Sorrenti, V. (2011) The DDAH/NOS pathway in human prostatic cancer cell lines: Antiangiogenic effect of L-NAME. *Int. J. Oncol. 39*, 1303−1310.

(13) Hess, D. T., and Stamler, J. S. (2012) Regulation by S-nitrosylation of protein post-translational modification. *J. Biol. Chem. 287*, 4411−4418.

(14) Nakamura, T., and Lipton, S. A. (2011) Redox modulation by S-nitrosylation contributes to protein misfolding, mitochondrial dynamics, and neuronal synaptic damage in neurodegenerative diseases. *Cell Death Differ. 18*, 1478−1486.

(15) Uehara, T., Nakamura, T., Yao, D., Shi, Z. Q., Gu, Z., Ma, Y., Masliah, E., Nomura, Y., and Lipton, S. A. (2006) S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. *Nature 441*, 513−517.

(16) Gaston, B., Sears, S., Woods, J., Hunt, J., Ponaman, M., McMahon, T., and Stamler, J. S. (1998) Bronchodilator S-nitrosothiol deficiency in asthmatic respiratory failure. *Lancet 351*, 1317−1319.

(17) Chung, K. K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J. C., Marsh, L., Dawson, V. L., and Dawson, T. M. (2004) S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science 304*, 1328−1331.

(18) van der Flier, A., and Sonnenberg, A. (2001) Function and interactions of integrins. *Cell Tissue Res. 305*, 285−298.

(19) Goel, H. L., Li, J., Kogan, S., and Languino, L. R. (2008) Integrins in prostate cancer progression. *Endocr.-Relat. Cancer 15*, 657−664.

(20) Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P., and Snyder, S. H. (2001) Protein S-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat. Cell Biol. 3*, 193−197.

(21) Lam, Y. W., Yuan, Y., Isaac, J., Babu, C. V., Meller, J., and Ho, S. M. (2010) Comprehensive identification and modified-site mapping of S-nitrosylated targets in prostate epithelial cells. *PLoS One 5*, e9075.

(22) Cress, A. E., Rabinovitz, I., Zhu, W., and Nagle, R. B. (1995) The $\alpha6\beta1$ and $\alpha6\beta4$ integrins in human prostate cancer progression. *Cancer Metastasis Rev. 14*, 219−228.

(23) Bonkhoff, H., Stein, U., and Remberger, K. (1993) Differential expression of $\alpha6$ and $\alpha2$ very late antigen integrins in the normal, hyperplastic, and neoplastic prostate: Simultaneous demonstration of cell surface receptors and their extracellular ligands. *Hum. Pathol. 24*, 243−248.

(24) Knox, J. D., Cress, A. E., Clark, V., Manriquez, L., Affinito, K. S., Dalkin, B. L., and Nagle, R. B. (1994) Differential expression of extracellular matrix molecules and the $\alpha6$-integrins in the normal and neoplastic prostate. *Am. J. Pathol. 145*, 167−174.

(25) Rabinovitz, I., Nagle, R. B., and Cress, A. E. (1995) Integrin $\alpha6$ expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype in vitro and in vivo. *Clin. Exp. Metastasis 13*, 481−491.

(26) Leung, Y. K., Mak, P., Hassan, S., and Ho, S. M. (2006) Estrogen receptor (ER)-β isoforms: A key to understanding ER-β signaling. *Proc. Natl. Acad. Sci. U.S.A. 103*, 13162−13167.

(27) Leung, Y. K., Lam, H. M., Wu, S., Song, D., Levin, L., Cheng, L., Wu, C. L., and Ho, S. M. (2010) Estrogen receptor $\beta2$ and $\beta5$ are associated with poor prognosis in prostate cancer, and promote cancer cell migration and invasion. *Endocr.-Relat. Cancer 17*, 675−689.

(28) Aumailley, M., Timpl, R., and Sonnenberg, A. (1990) Antibody to integrin $\alpha6$ subunit specifically inhibits cell-binding to laminin fragment 8. *Exp. Cell Res. 188*, 55−60.

(29) Sonnenberg, A., Linders, C. J., Modderman, P. W., Damsky, C. H., Aumailley, M., and Timpl, R. (1990) Integrin recognition of different cell-binding fragments of laminin (P1, E3, E8) and evidence that $\alpha6\beta1$ but not $\alpha6\beta4$ functions as a major receptor for fragment E8. *J. Cell Biol. 110*, 2145−2155.

(30) Lam, H. M., Suresh Babu, C. V., Wang, J., Yuan, Y., Lam, Y. W., Ho, S. M., and Leung, Y. K. (2012) Phosphorylation of human estrogen receptor-β at serine 105 inhibits breast cancer cell migration and invasion. *Mol. Cell. Endocrinol. 358*, 27−35.

(31) Neumann, M., Klar, S., Wilisch-Neumann, A., Hollenbach, E., Kavuri, S., Leverkus, M., Kandolf, R., Brunner-Weinzierl, M. C., and Klingel, K. (2011) Glycogen synthase kinase-3β is a crucial mediator of signal-induced RelB degradation. *Oncogene 30*, 2485−2492.

(32) Chung, L. W., Baseman, A., Assikis, V., and Zhau, H. E. (2005) Molecular insights into prostate cancer progression: The missing link of tumor microenvironment. *J. Urol. 173*, 10−20.

(33) Thomazzi, S. M., Ferreira, H. H., Conran, N., De, N. G., and Antunes, E. (2001) Role of nitric oxide on in vitro human eosinophil migration. *Biochem. Pharmacol. 62*, 1417−1421.

(34) Orucevic, A., Bechberger, J., Green, A. M., Shapiro, R. A., Billiar, T. R., and Lala, P. K. (1999) Nitric-oxide production by murine mammary adenocarcinoma cells promotes tumor-cell invasiveness. *Int. J. Cancer 81*, 889−896.

(35) Foster, M. W., Hess, D. T., and Stamler, J. S. (2009) Protein S-nitrosylation in health and disease: A current perspective. *Trends Mol. Med. 15*, 391−404.

(36) Thom, S. R., Bhopale, V. M., Mancini, D. J., and Milovanova, T. N. (2008) Actin S-nitrosylation inhibits neutrophil $\beta2$ integrin function. *J. Biol. Chem. 283*, 10822−10834.

(37) Rahman, M. A., Senga, T., Ito, S., Hyodo, T., Hasegawa, H., and Hamaguchi, M. (2010) S-nitrosylation at cysteine 498 of c-Src tyrosine kinase regulates nitric oxide-mediated cell invasion. *J. Biol. Chem. 285*, 3806−3814.

(38) Kim, S. F., Huri, D. A., and Snyder, S. H. (2005) Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. *Science 310*, 1966−1970.
(39) Hess, D. T., Matsumoto, A., Kim, S. O., Marshall, H. E., and Stamler, J. S. (2005) Protein S-nitrosylation: Purview and parameters. *Nat. Rev. Mol. Cell Biol. 6*, 150−166.
(40) Felley-Bosco, E., Bender, F., and Quest, A. F. (2002) Caveolin-1-mediated post-transcriptional regulation of inducible nitric oxide synthase in human colon carcinoma cells. *Biol. Res. 35*, 169−176.
(41) Echarri, A., Muriel, O., and Del Pozo, M. A. (2007) Intracellular trafficking of raft/caveolae domains: Insights from integrin signaling. *Semin. Cell Dev. Biol. 18*, 627−637.
(42) Salanueva, I. J., Cerezo, A., Guadamillas, M. C., and Del Pozo, M. A. (2007) Integrin regulation of caveolin function. *J. Cell. Mol. Med. 11*, 969−980.
(43) Miyamoto, S., Teramoto, H., Gutkind, J. S., and Yamada, K. M. (1996) Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: Roles of integrin aggregation and occupancy of receptors. *J. Cell Biol. 135*, 1633−1642.
(44) Ahamed, J., Versteeg, H. H., Kerver, M., Chen, V. M., Mueller, B. M., Hogg, P. J., and Ruf, W. (2006) Disulfide isomerization switches tissue factor from coagulation to cell signaling. *Proc. Natl. Acad. Sci. U.S.A. 103*, 13932−13937.
(45) Stathakis, P., Fitzgerald, M., Matthias, L. J., Chesterman, C. N., and Hogg, P. J. (1997) Generation of angiostatin by reduction and proteolysis of plasmin. Catalysis by a plasmin reductase secreted by cultured cells. *J. Biol. Chem. 272*, 20641−20645.
(46) Lahav, J., Gofer-Dadosh, N., Luboshitz, J., Hess, O., and Shaklai, M. (2000) Protein disulfide isomerase mediates integrin-dependent adhesion. *FEBS Lett. 475*, 89−92.
(47) Walsh, G. M., Leane, D., Moran, N., Keyes, T. E., Forster, R. J., Kenny, D., and O'Neill, S. (2007) S-Nitrosylation of platelet αIIbβ3 as revealed by Raman spectroscopy. *Biochemistry 46*, 6429−6436.
(48) Walsh, G. M., Sheehan, D., Kinsella, A., Moran, N., and O'Neill, S. (2004) Redox modulation of integrin [correction of integin] αIIbβ3 involves a novel allosteric regulation of its thiol isomerase activity. *Biochemistry 43*, 473−480.
(49) Lahav, J., Wijnen, E. M., Hess, O., Hamaia, S. W., Griffiths, D., Makris, M., Knight, C. G., Essex, D. W., and Farndale, R. W. (2003) Enzymatically catalyzed disulfide exchange is required for platelet adhesion to collagen via integrin α2β1. *Blood 102*, 2085−2092.
(50) King, T. E., Pawar, S. C., Majuta, L., Sroka, I. C., Wynn, D., Demetriou, M. C., Nagle, R. B., Porreca, F., and Cress, A. E. (2008) The role of α6 integrin in prostate cancer migration and bone pain in a novel xenograft model. *PLoS One 3*, e3535.