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Nitric Oxide Increases Lysine 48-Linked Ubiquitination Following Arterial Injury

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Abstract

Introduction—Proteins are targeted for degradation by the addition of a polyubiquitin chain. Chains of ubiquitin linked via lysine 48 (K48) are associated with protein degradation while chains linked via lysine 63 (K63) are associated with intracellular signaling. We have previously shown that nitric oxide (NO) inhibits neointimal hyperplasia in association with increasing the ubiquitination and degradation of UbcH10. The aim of this study is to characterize the effect of arterial injury and NO on K48- or K63-linked ubiquitination of cellular proteins.

Methods—The rat carotid artery balloon injury model was performed. Treatment groups included control, injury, injury+proline NONOate (PROLI/NO), and PROLI/NO alone. Arteries were harvested at designated time points and sectioned for immunohistochemical analysis of K48- and K63-linked ubiquitination or homogenized for protein analysis. Vascular smooth muscle cells (VSMC) harvested from rat aortae were exposed to the NO donor diethylenetriamine NONOate (DETA/NO). Protein expression was determined by Western blot analysis, or immunoprecipitation and Western blot analysis.

Results—Arterial injury increased K48-linked ubiquitination *in vivo*. The addition of PROLI/NO following injury caused a further increase in K48-linked ubiquitination at 1 and 3 days, however, levels returned to that of injury alone by 2 weeks. Interestingly, treatment with PROLI/NO alone increased K48-linked ubiquitination *in vivo* to levels similar to injury alone. There were minimal changes in K63-linked ubiquitination in all three treatment groups. DETA/NO increased K48-linked ubiquitination in VSMC *in vitro* but had minimal effects on K63-linked ubiquitination. Low doses of DETA/NO decreased K48-linked ubiquitination of cyclin A and B, while high doses of DETA/NO increased K48-linked ubiquitination of cyclin A and B. Minimal changes were seen in K63-linked ubiquitination of cyclin A and B *in vitro*.

Conclusions—Arterial injury and NO increased K48-linked ubiquitination *in vivo* and *in vitro*. Remarkably, minimal changes were seen in K63-linked ubiquitination. These novel findings provide important insights into the vascular biology of arterial injury and suggest that one mechanism by which NO may prevent neointimal hyperplasia is through regulation of protein ubiquitination.

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Introduction

The ubiquitin-proteasome system is responsible for the breakdown of the majority of short-lived proteins in the eukaryotic cell, including proteins involved in maintaining normal cell cycle progression.[1;2] Protein ubiquitination proceeds via the coordinated activities of the ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) enzymes, initially linking an ϵ -amino group of the C-terminal glycine of ubiquitin to a lysine on a protein targeted for degradation.[3] Subsequently, a polyubiquitin chain is formed by linking a ubiquitin molecule to a lysine on the end ubiquitin molecule of the growing chain. Since the ubiquitin molecule has 7 lysine residues, several linkage sites are available for extension of the ubiquitin chain. Depending on which lysine residue is used to link the ubiquitin molecules, the target protein has a different cellular fate. Two of the best-studied ubiquitin linkages are lysine 48 (K48) and lysine 63 (K63). A K48-linked polyubiquitin chain is associated with proteins marked for degradation by the 26S proteasome.[4;5] In contrast, K63-linked ubiquitination is associated with non proteolytic intracellular signaling.[4]

Neointimal hyperplasia is a complication of vascular interventions. It is characterized by aggressive cellular proliferation and occurs following interventions to restore flow to occluded arteries, such as angioplasty and stenting, endarterectomy, and bypass grafting. We, and others, have shown that nitric oxide (NO) causes G₀/G₁ cell cycle arrest, prevents vascular smooth muscle cell (VSMC) proliferation, and is effective at inhibiting the formation of neointimal hyperplasia in small and large animal models of arterial injury and vein bypass grafting.[6–15] We have previously shown that levels of the E2 enzyme, UbcH10, which is known to regulate the degradation of numerous cell cycle proteins, directly correlates with cellular proliferation. Additionally we have shown that levels of UbcH10 are increased after arterial balloon injury, and that NO applied after injury decreases UbcH10 levels through increasing its ubiquitination, and hence degradation[16]. Given the importance of the ubiquitination and degradation of cell cycle proteins for the progression of the cell cycle, we hypothesize that arterial injury will increase K48-linked ubiquitination of cellular proteins. Additionally, given the efficacy of NO in preventing neointimal hyperplasia and our prior data showing that NO increases ubiquitination of UbcH10, we hypothesize that NO will increase K48-linked ubiquitination of cellular proteins and have minimal effect on K63-linked ubiquitination.

Materials and Methods

Cell culture

VSMC were harvested and cultured from the abdominal aorta of Sprague-Dawley rats (Harlan, Indianapolis, IN), and maintained as previously described.[9;17] Cultured cells had the characteristic appearance of hills and valleys and were routinely more than 95% pure by smooth muscle cell α -actin staining.[18]

NO donor preparation

The NO donors proline NONOate (PROLI/NO) and diethylenetriamine NONOate (DETA/NO) were synthesized as previously described.[19;20] Stock solutions (10 mM) were prepared by dissolving solid DETA/NO in the appropriate complete culture medium just before use. PROLI/NO was used in the powdered form.

Western blot analysis

Whole-cell suspensions of DETA/NO-treated and untreated VSMC were prepared as previously described [21] and protein concentrations determined by bicinchoninic acid assays were performed according to the manufacturer's instructions (Pierce, Rockford, IL).

Whole-cell suspensions were subjected to polyacrylamide gel electrophoresis on 12% gels, after which proteins were transferred to a nitrocellulose membrane. Protein levels were determined using primary antibodies to K48- and K63-linked ubiquitin (1:500, Millipore, Bedford, MA). The membranes were then rinsed before adding the appropriate secondary antibody at a concentration suggested by the manufacturer for 1 hour. Protein levels were visualized using chemoluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and imaged with the Kodak Image Station in vivo FX (Eastman Kodak, Rochester, NY). Densitometry was performed on all Western blots using Image J software and normalized to control groups.

Immunoprecipitation

For immunoprecipitations, DETA/NO-treated and untreated VSMC were lysed using lysis buffer A (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycoltetraacetic acid (EGTA), 1% (v/v) Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, 1 mM sodium orthovanadate, 1 μ g/mL leupeptin, and 1 mM PMSF), and 50 μ g of lysate was incubated with 1 μ g of goat IgG plus 20 μ L of protein A/G sepharose (both from Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at 4°C on a rotating wheel. The beads were then pelleted by centrifugation for 5 minutes at 1500 rpm and 4°C in an Eppendorf 5417R centrifuge (Westbury, NY), and the supernatant transferred to a fresh tube, to which was added 20 μ L of protein A/G sepharose and 1:100 or 1:50 dilution of K48- or K63-linked ubiquitin antibody, respectively (Millipore, Bedford, MA). After overnight incubation at 4°C on a rotating wheel, the beads were pelleted at 2500 rpm for 15 minutes at 4°C and the supernatants carefully removed. The beads were resuspended in Laemmli loading buffer and boiled at 95°C. The beads were then pelleted by centrifugation for 5 minutes at 1500 rpm. The supernatant fraction was subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and protein levels were detected using primary antibodies to cyclin A and B (1:500; Abcam, Cambridge, MA). The membranes were then rinsed before adding the appropriate secondary antibody at a concentration suggested by the manufacturer for 1 hour. Protein levels were visualized using chemoluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and imaged with the Kodak Image Station in vivo FX (Eastman Kodak, Rochester, NY). Densitometry was performed on all Western blots using Image J software and normalized to control groups.

Animal surgery

All animal procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication 85–23, 1996) and approved by the Northwestern University Animal Care and Use Committee. Male Sprague Dawley rats weighing between 350–400 g were anesthetized with inhaled isoflurane (0.5–3%). Atropine was administered subcutaneously (0.1 mg/kg) to decrease airway secretions. After a midline neck incision, the left common carotid artery (CCA), external carotid artery (ECA), and the internal carotid artery (ICA) were dissected and proximal and distal control obtained with microclips. A transverse arteriotomy was created on the ECA. A 2 French Fogarty catheter (generously provided by Edwards Lifesciences) was inserted into the CCA through the ECA, and the CCA injured by inflating the balloon to 5 atmospheres of pressure for 5 minutes. Following injury, the catheter was removed, the ECA ligated, and flow restored to the CCA and ICA, as previously described.[11;12;22] Immediately after injury and restoration of flow, 20 mg of PROLI/NO powder was applied evenly to the periadventitial surface of the injured CCA of rats in the treatment group as previously described.[11;12;22] The neck incision was closed and carotid arteries were harvested at 2 hours, 1 day, 3 days, or 14 days post injury. Treatment groups included no

injury, injury alone, injury + PROLI/NO, and PROLI/NO alone. Of note, the addition of 20 mg of PROLI/NO releases approximately 5.1×10^3 nmol/mm².

Tissue processing for lysates

For carotid lysates (n=7=8/group), arteries were harvested 2 hours, 24 hours, or 72 hours after balloon injury. Rats were anesthetized, euthanized, and the vessels exposed as described above; however, the rats did not undergo *in situ* perfusion and fixation. Each carotid artery was ligated at the aortic arch and explanted *en bloc* as quickly as possible. Vessels were identified, washed in cold 1X PBS, opened *en face*, and the injured section of the CCA was excised, snap-frozen, and stored in liquid nitrogen until lysed via mechanical means (ceramic mortar and pestle, CoorsTek; Golden, CO) and resuspended in lysis buffer (50 mM Hepes [pH 7.5], 150 mM NaCl, 10% glycerol (v/v), 10 mM sodium pyrophosphate [Na₄P₂O₇], 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid [EGTA], 1% Triton X-100, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 50 mM sodium fluoride [NaF], 1 mM sodium orthovanadate [Na₃VO₄], and 1 mM PMSF). Protein concentration was determined via BCA assay, and samples were stored at -80°C until Western blot analysis.

Tissue processing for histology

Carotid arteries were harvested 14 days after balloon injury as follows. This time point was selected to allow significant neointimal hyperplasia formation, since luminal narrowing following arterial injury is most pronounced at this time point[23]. Rats were anesthetized with isoflurane and euthanized with 0.5 mL euthasol, and a bilateral thoracotomy was performed. A long midline incision was made to expose both carotid arteries. Vessels were perfused and fixed *in situ* using cold 1X PBS (250 mL) and 2% paraformaldehyde (500 mL), and then explanted *en bloc* from the aortic arch to the carotid bifurcation. The injured segment of the carotid arteries were frozen in TissueTek O.C.T. compound (Sakura Finetek USA, Torrance, CA) and cut into 5-µm sections throughout the entire injured area, as previously described.[11;12;22]

Immunohistochemistry tissue staining

Carotid artery sections of uninjured, injured, and NO-treated rats (n=3/group) were fixed for 5 minutes in ice cold acetone. Sections were then permeabilized with 0.3% Triton X-100 for 20 minutes and incubated in 0.3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidases. After blocking with horse serum (Sigma, St. Louis, MO) for 30 minutes, primary antibody to K48- or K63-linked ubiquitin (Millipore) was diluted 1:50 in 0.5% BSA and added for 1 hour. Next, after rinsing, biotinylated secondary antibody diluted 1:1000 in PBS was added for 1 hour, followed by rinsing and the addition of streptavidin-bound horseradish peroxidase for 30 minutes. The stain was developed by incubation in the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 90 seconds, and a counterstain of hematoxylin was applied. Coverslips were affixed with Permount, and allowed to dry overnight. For each type of staining, six equally-spaced sections from the area of injury were stained from each animal. Digital images of stained sections were collected with light microscopy using an Olympus BHT microscope (Melville, NY) with 4X, 10X and 40X objectives.

Blinded grading and statistical analysis

Images of the stained sections were assessed by blinded graded of the intensity of staining in the neointima and adventitia of each section on a scale from 0–3. Results are expressed as the mean ± standard error (SE) of the mean. Differences between groups were analyzed by one-way analysis of variance (ANOVA) (SigmaStat; SPSS. Chicago, IL). Statistical significance was assumed for P<0.05.

Results

Injury and NO both increased K48-linked ubiquitination *in vivo*

To begin our investigation into the effects of NO on K48- and K63-linked ubiquitination, we performed Western blotting analysis on carotid artery lysates of animals subjected to the carotid artery injury model. As seen in Figure 1, at 24 and 72 hours post injury, K48-linked ubiquitination increased following arterial injury by 72% and 15%, respectively. At all time points, NO further increased K48-linked ubiquitination following injury to levels above those seen with injury alone, by 58%, 90%, and 63% relative to control at 2, 24, and 72 hours. In contrast, a decrease in K63-linked ubiquitination was observed after injury or injury + PROLI/NO.

We continued our investigation by performing immunohistochemical analysis of carotid artery sections 2 weeks following arterial injury. As seen in Figure 2, injury dramatically increased K48-linked ubiquitination in the neointima and adventitia. This increase persisted in the injury + PROLI/NO treatment group. Interestingly, the animals that received PROLI/NO alone with no injury show a dramatic increased K48-linked ubiquitination, similar to that of injury alone. Once again, minimal changes in K63-linked ubiquitination were seen after injury, injury + PROLI/NO, or PROLI/NO alone.

To quantify the K48-linked ubiquitin staining, blinded grading of the stained sections was performed by layer. As seen in Figure 3A, a statistically significant increase in K48-linked ubiquitin staining was observed in the neointima between control (0.4 ± 0.5) versus injury (3 ± 0.0 , $P < 0.001$), injury + PROLI/NO (2.2 ± 0.7 , $P < 0.001$), and PROLI/NO alone (2.4 ± 0.5 , $P < 0.001$). As seen in Figure 3B, a statistically significant increase in K48-linked ubiquitin staining was observed in the adventitia between control (0.0 ± 0.0) versus injury (2.1 ± 0.6 , $P < 0.001$), injury + PROLI/NO (2.4 ± 0.5 , $P < 0.001$), and PROLI/NO alone (2.8 ± 0.4 , $P < 0.001$). In the neointima, there was a statistically significant difference in K48-linked ubiquitin staining between injury alone versus injury + NO ($P < 0.007$), however there was no statistically significant difference in the adventitia. These data suggest that both injury and NO increase K48-linked ubiquitination of cellular proteins, but both have small effects on K63-linked ubiquitination of cellular proteins.

NO increased K48-linked ubiquitination *in vitro*

To continue our investigation *in vitro*, we performed Western blotting analysis on extracts of VSMC treated in the presence or absence of the NO donor DETA/NO (0.5 and 1 mM) for 24 hours. As seen in Figure 4, DETA/NO increased K48-linked ubiquitination at both 0.5 and 1.0 mM, by 37% and 48% respectively. At 0.5 mM DETA/NO, NO increased K63-linked ubiquitination by 25%, while 1.0 mM DETA/NO decreased K63-linked ubiquitination by 44%. These data correspond with our *in vivo* findings, supporting the fact that NO increases K48-linked ubiquitination of cellular proteins, while having lesser or opposite effects on K63-linked ubiquitination.

NO has a bimodal effect on K48-linked ubiquitination of cyclin A and cyclin B in VSMC

To investigate the effect of NO on the ubiquitination of specific cell cycle proteins, we assessed the K48- and K63-linked ubiquitination of cyclins A and B, which are both ubiquitinated by UbcH10, in VSMC treated with and without the NO donor DETA/NO. We immunoprecipitated all K48- and K63-linked ubiquitin chains and performed Western blot analysis for cyclin A and B. As seen in Figure 5, 0.5 mM DETA/NO decreased the K48-linked ubiquitination of both cyclin A and B, by 52% and 58%, respectively. However, 1 mM DETA/NO increased the K48-linked ubiquitination of cyclin A and cyclin B, by 30% and 24%, respectively. In contrast, 1mM DETA/NO decreased K63-linked ubiquitination of

cyclin A and B by 4% and 44%, respectively. These data suggest that low doses of NO decrease K48-linked ubiquitination of cyclin A and B, while higher doses of NO increase K48-linked ubiquitination of cyclin A and B. In contrast, K63-linked ubiquitination of cyclin A and B was decreased by higher doses of NO. These findings correspond with our *in vivo* data regarding the effects of NO on K48- and K63-linked ubiquitination of cellular proteins.

Discussion

We have shown that arterial injury increases K48-linked ubiquitination of cellular proteins, particularly in the neointima and adventitia, but had lesser or opposite effects on K63-linked ubiquitination. We found that periadventitial administration of NO, following injury or without injury, also increased K48-linked ubiquitination of cellular proteins. Our *in vitro* experiments corroborated our *in vivo* findings. Administration of NO increased K48-linked ubiquitination of cellular proteins in VSMC and had lesser or opposite effects on K63-linked ubiquitination. Immunoprecipitation of K48-linked ubiquitinated cell cycle proteins from VSMC showed a bimodal effect of NO on K48-linked ubiquitination. At lower concentrations, NO decreased K48-linked ubiquitination of cyclin A and B. While higher concentrations of NO increased K48-linked ubiquitination of cyclin A and B, K63-linked ubiquitination of cyclin A and B was decreased at these levels.

Our findings suggest that increased K48-linked ubiquitination of cellular proteins is an integral process that occurs in association with the development of neointimal hyperplasia. Much is known about the importance of K48-linked ubiquitination in cellular proliferation. Mutation of the lysine 48 residue of ubiquitin to arginine in *S. cerevisiae* results in cell cycle arrest in the G₂, M, and G₁ phases, suggesting that K48-linked ubiquitination is essential for cell cycle progression.[24] Our data is in alignment with this, and to our knowledge, this is the first study suggesting a role for increased K48-linked ubiquitination in the pathogenesis of neointimal hyperplasia. Glotzer et al. showed that degradation of the cyclins is dependent on ubiquitination and subsequent proteasomal degradation.[25] The increased VSMC proliferation seen in neointimal hyperplasia may be caused by accelerated progression through the cell cycle due to increased K48-linked ubiquitination of the cyclins.

In VSMC, we observed a consistent increase in K48-linked ubiquitination at both 0.5 mM and 1 mM DETA/NO. In contrast, we witnessed lesser or opposite effects on K63-linked ubiquitination. These data, especially at 1 mM, suggest that an increase in K48-linked ubiquitination seen with NO is unique, and not observed with other residues such as the K63 residue. Similar effects were seen *in vivo*, with a large increase in K48-linked ubiquitination after administration of NO, compared with the lesser or opposite changes observed in K63-linked ubiquitination. It is possible that NO has different effects on K63-linked ubiquitination, but we do not have enough data to determine the consequences of the change in K63-linked ubiquitination seen with NO treatment.

Similar to our earlier data showing that NO increased ubiquitination and degradation of UbcH10, we found that high doses of NO resulted in increased K48-linked ubiquitination of cyclin A and B. While increased K48-linked ubiquitination of cyclin A and B would be expected to increase cellular proliferation, K48-linked ubiquitination is responsible for decreasing cellular proliferation in other pathways. For example, K48-linked ubiquitination is essential for the degradation of the nuclear protein BCL-3, which has been implicated in many solid and hematologic malignancies.[26] Additionally, K48-linked ubiquitination is required for the degradation of the oncoprotein Myc.[27] NO could be directly preventing neointimal hyperplasia by increasing the K48-linked ubiquitination and degradation of other

cellular proteins besides cyclin A and B, with the overall balance of pro- and anti-proliferative cell cycle proteins playing an important role.

We have previously shown that the levels of cyclin B, but not cyclin A in VSMC, are decreased by NO *in vitro*. [28] This observation supports our finding that NO increases the K48-linked ubiquitination of cyclin B. However, it would be expected that levels of cyclin A would also be decreased by NO. One possibility is that NO has been shown to inhibit the 26S proteasome itself, [29;30] preventing the degradation of cyclin A after it is ubiquitinated via K48-linked chains. Additionally, while cyclin A is ubiquitinated, this modification is reversible, so the proteins may not necessarily be degraded, but merely prevented from binding its cyclin dependent kinase partner. Lastly, other cell cycle control pathways might be involved in counteracting the increased K48-linked ubiquitination of the cyclins following NO treatment, to prevent excessive degradation of cyclin A.

A limitation of our study is that the precise mechanism by which NO affects K48-linked ubiquitination remains unknown. Previous studies have shown that NO inhibits the 26S proteasome. [29;30] Since the proteasome is responsible for degrading K48-linked ubiquitinated proteins, the inhibition of the proteasome by NO could be causing a buildup of these ubiquitinated proteins. In addition, we observed a decrease in K48-linked ubiquitination of cyclins A and B at low doses of NO but an increase at high doses. Perhaps NO has both direct and indirect effects. It is possible that NO might act directly to decrease K48-linked ubiquitination of cyclin A and B at low doses by affecting the E2 enzymes involved in mediating K48 linkages of these proteins, such as UbcH10. Yet, at higher doses this effect may be mediated indirectly through inhibition of the proteasome as discussed above. Additional studies are necessary to determine the precise mechanism by which NO affects K48-linked ubiquitination of cellular proteins, and how this effect impacts cellular proliferation and neointimal hyperplasia, as this has never before been studied.

In conclusion, we showed that K48-linked ubiquitination is increased in neointimal hyperplasia. Additionally, we showed that administration of NO both *in vivo* and *in vitro* resulted in an increase in K48-linked ubiquitination of cellular proteins, and specifically of cyclin A and B. While further study is warranted to delineate the mechanism by which NO imparts these effects, our results provide valuable insights into the pathogenesis of neointimal hyperplasia and the effects of NO on protein ubiquitination. This important work could lead to the development of a therapy targeting the K48-linked ubiquitination of cellular proteins to prevent neointimal hyperplasia.

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Figure 1.

A) Carotid arterial injury and nitric oxide (NO) treatment both increased K48-, but not K63-linked ubiquitination *in vivo*. Following balloon injury and treatment with or without the NO donor proline NONOate (PROLI/NO, 20 mg, t = 2, 24, or 72 hours), rat carotid arteries were homogenized and subjected to Western blot analysis using antibodies to K48- or K63-linked ubiquitin. Injury increased K48-linked ubiquitination at 24 and 72 hours. Treatment with NO following injury further increased K48-linked ubiquitination at all time points. In contrast, decreases in K63-linked ubiquitination following injury or NO treatment are seen.

B) Graphical representation of the densitometry analysis normalized to the control group. n=7–8/treatment group.

Figure 2.

Carotid artery injury and nitric oxide (NO) treatment increased K48-, but not K63-linked ubiquitination *in vivo*. Following balloon arterial injury and treatment with or without PROLI/NO(20 mg, t = 14 days), or treatment with PROLI/NO alone, rat carotid artery cross sections underwent immunohistochemistry staining 2 weeks after injury for K48- and K63-linked ubiquitin (brown). K48-linked ubiquitination increased following arterial injury, injury + NO treatment, or NO alone. Minimal changes were seen following injury or NO treatment in K63-linked ubiquitination. n=3/treatment group. Negative control reflects staining with saline substituted for the primary antibody.

Figure 3.

Carotid injury and nitric oxide (NO) treatment increased K48-linked ubiquitination *in vivo*. Blinded grading of neointimal and adventitial immunohistochemical staining for K48-linked ubiquitin chains shows that injury, injury + NO, and NO alone all significantly increase K48-linked ubiquitin staining relative to control arteries at 2 weeks (* $P < 0.001$ vs. uninjured group; ** $P < 0.007$ vs. injury alone group). $n=3$ /treatment group.

Figure 4.

A) Nitric oxide (NO) increased K48-, but not K63-linked ubiquitination *in vitro*. Vascular smooth muscle cells (VSMC) were treated with the NO donor diethylenetriamine NONOate (DETA/NO, 0.5–1 mM), then subjected to Western blot analysis using antibodies to K48- or K63-linked ubiquitin. NO caused an increase in K48-linked ubiquitination at both concentrations. Lesser or opposite changes were seen in K63-linked ubiquitination. B) Graphical representation of the densitometry analysis normalized to the control group. $t = 24$ hours. Images are representative of three separate experiments.

Figure 5.

A) Nitric oxide (NO) has a bimodal effect on K48-, but not K63-linked ubiquitination of cyclins A and B. VSMC were treated with DETA/NO (0.5–1 mM). Immunoprecipitation (IP) was performed using antibodies to K48- or K63-linked ubiquitin. Immunoprecipitated proteins were then subjected to Western blot (WB) analysis using antibodies to cyclin A or B. While 0.5 mM DETA/NO decreased K48-linked ubiquitination, 1 mM DETA/NO increased K48-linked ubiquitination. 1 mM DETA/NO decreased K63-linked ubiquitination. B) Graphical representation of the densitometry analysis normalized to the control group. Images are representative of two separate experiments.