High glucose attenuates protein S-nitrosylation in endothelial cells: role of oxidative stress

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Running title: High glucose attenuates S-nitrosylation

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Abstract

**Objective.** Hyperglycemia-induced endothelial dysfunction, via a defect of nitric oxide (NO) bioactivity and overproduction of superoxide, is regarded as one of the most significant events contributing to the vascular lesions associated with diabetes mellitus. However, the mechanisms underlying such hyperglycemic injury remain undefined. We hypothesized that alterations in cellular protein S-nitrosylation may contribute to hyperglycemia-induced endothelial dysfunction.

**Research Design and Methods.** We exposed endothelial cells to high glucose in the presence and absence of reactive oxygen species inhibitors and used the biotin switch assay to analyze the alteration in the global pattern of protein S-nitrosylation, compared to cells cultured under normal glucose conditions. We identified endogenous S-nitrosylated proteins by mass spectrometry and/or immunoblotting with specific antibodies.

**Results.** High glucose treatment induced a significant reduction of endogenous S-nitrosylated proteins that include eNOS, β-actin, vinculin, diacylglycerol kinase α, GRP78, ERK-1 and transcription factor NF-κB. Interestingly, these changes were completely reversed by inhibition of superoxide production, suggesting a key role for oxidative stress in the regulation of S-nitrosylation under hyperglycemic conditions. In addition, we found that in parallel with the restoration of decreased S-nitrosylation of NF-κB, high glucose-induced NF-κB activation was blocked by the superoxide inhibitors.

**Conclusions.** The alterations in protein S-nitrosylation may underlie the adverse effect of hyperglycemia on the vasculature, such as endothelial dysfunction and the development of diabetic vascular complications.

**Key words:** diabetes, endothelial cells, hyperglycemia, oxidative stress, S-nitrosylation.
Hyperglycemia is now well recognized as a major etiological factor causing both micro- and macro-vascular lesions associated with diabetes mellitus (1;2). A series of interconnected biochemical changes initiated by hyperglycemia have been documented to directly affect cellular function resulting in abnormal vascular remodelling and the development of diabetic complications (3;4). Endothelial dysfunction, characterized by a loss of nitric oxide (NO) bioactivity via increased superoxide production, is regarded as one of the most important cellular events accounting for the adverse effects of high glucose on the vasculature (4).

NO is constitutively expressed by endothelial cells, serving as a pivotal endothelium-derived modulator that maintains normal function of the vasculature through its vasodilator, anti-platelet, anti-proliferative, antioxidant and anti-inflammatory properties. Indeed, a decline in NO bioactivity and the resultant endothelial dysfunction occurs in many disease settings, including hyperlipidemia, hypertension, metabolic syndrome and diabetes mellitus (reviewed (5)). Substantial evidence exists from both in vitro and in vivo studies showing that exposure to hyperglycemia reduces endothelial NO availability and its bioactivity (6;7). High glucose-induced NO deficiency is partially attributable to the uncoupling of the endothelial nitric oxide synthase (eNOS) reaction resulting in the net synthesis of superoxide and to the oxidative inactivation of NO (8). However, the downstream signaling events augmented by the reduction of NO bioavailability under high glucose conditions remain largely unexplored.

The interaction of NO with heme containing proteins (such as in guanylyl cyclase) is the most recognized signaling event associated with NO, whilst it is also increasingly apparent that nitrosylation of protein sulfhydryl groups represents an important additional NO-dependent signaling mechanism (9). To date, over 100 proteins with a broad functional spectrum have been identified as targets for S-nitrosylation (10). S-nitrosylation entailing the transfer of nitrosonium to reduced cysteine residue, has been documented to regulate the activity of a number of metabolic enzymes, proteases, protein kinases/phosphatases, receptors, transcription factors, together with various cytoskeletal and structural components (10). It has been reported that S-nitrosylation of proteins in endothelial cells occurs under various physiological or pathophysiological conditions and plays an important role in the regulation of endothelial function (11). For instance, treatment with the pro-inflammatory cytokine TNFα or oxidized low density lipoprotein (oxLDL) resulted in a decrease in endothelial protein S-nitrosylation (12). Exposure to shear stress, which is vasoprotective, resulted in a global increase in protein S-nitrosylation in endothelial cells (13).

Despite current knowledge that a decline in NO bioactivity accounts for the effect of high glucose on endothelial dysfunction and vascular injury associated with diabetes, the molecular mechanisms underlying such events remain undefined. The studies described herein were undertaken to examine whether S-nitrosylation of proteins is affected by high glucose in endothelial cells, together with an examination of potential mechanisms involved in the regulation of
protein S-nitrosylation under such conditions.

**Research Design and Methods**

**Cell culture and Reagents.**

Reagents not otherwise indicated were from Sigma (St Louis, MO). Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously (14). Cells were used at passages 2 to 3. The cells were grown in M199 supplemented with 20% FCS and endothelial growth factors. For the experimental studies, HUVEC were allowed to reach confluence in the regular growth media and then cultured for 3 days in (i) media containing 5.5 mmol/L glucose (referred as normal glucose, NG); (ii) NG medium supplemented with additional glucose to final concentration of 30 mmol/L (high glucose); or (iii) NG medium containing 24.5 mmol/L mannitol. For experiments including reactive oxygen species (ROS) inhibitors the cells were incubated overnight with the NAD(P)H oxidase (NOX) inhibitor apocynin (500 \( \mu \)M), NOX and nitric oxide synthase (NOS) inhibitor diphenyleneiodonium (DPI) (10 \( \mu \)M), superoxide dismutase mimetic TEMPOL (1mM) or with the mitochondrial electron transport chain (mETC) inhibitors carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (500 nM), thenoyltrifluoroacetone (TTFA) (10 \( \mu \)M) or alpha-cyano-4-hydroxycinnamic acid (4-OHCA) (250 \( \mu \)M) and compared to DMSO treated control. All ROS inhibitors were dissolved in DMSO and diluted 1: 1000 in culture medium when added to the cells. In some experiments the cell extracts were incubated with 100 \( \mu \)M reduced glutathione (GSH) or nitrosylated glutathione (GSNO) 100 \( \mu \)M for 20 min at room temperature in the dark.

**Detection of S-nitrosylated proteins by the biotin switch assay.**

The biotin switch assay was performed essentially as described in (15) with some modifications. After treatment, cells were lysed in HEN buffer (250 mM Hepes, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine) containing 0.1% SDS. Cellular extracts were adjusted to below 0.8 mg/ml of protein and equal amounts were incubated with 4 volumes of HEN buffer containing 2.5% SDS, and 20 mM S-methyl methanethiosulfonate (MMTS) at 50°C for 20 min with frequent agitation to block free thiols. After blocking, extracts were precipitated with 4 volumes of cold (-20°C) acetone, dried at room temperature and resuspended in 100\( \mu \)l of HENS buffer (HEN plus 1% SDS) adjusted to pH 6.8 to prevent the biotinylation of primary amines. Until this point all steps were carried out in the dark. S-nitrosothiols (SNO) were decomposed by adding 5 mM ascorbate followed by incubation with 2 mM biotin-BMCC (Pierce, Rockford, IL) or vehicle alone for 2 hrs at room temperature. Proteins were then precipitated again using acetone and resuspended in HENS buffer. For purification of biotinylated proteins, samples were diluted with two volumes of neutralization buffer (20 mM Hepes, pH 7.7, 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100) and 25 \( \mu \)l of a 50% streptavidin agarose suspension (Sigma) and incubated for 1 hr at room temperature with agitation. Beads were washed 4 times with neutralization buffer. Proteins were eluted by boiling in reducing sample buffer for 5 mins and separated on 10% SDS-PAGE gels, transferred to PVDF and blotted with specific antibodies, including anti-biotin.
(Sigma), anti-actin (Chemicon Int.), anti-vinculin (Chemicon, Temecula, CA), anti-talin (Upstate, Lake Placid NY), anti-vimentin (Promega, Madison, WI) and anti-p50 NF-κB (Upstate Lake Placid, NY), respectively. Developed using HRP conjugated secondary antibody (Abcam, Cambridge, UK) and ECL (Amersham Pharmacia Biotech). Band intensities in the blots were analyzed by densitometry using an ImageQuant program (Molecular Dynamics) and normalized to control bands.

**Immunostaining of S-Nitrosylated Proteins.**

Cells were incubated with 5.5 or 30 mmol/L glucose for 3 days and fixed with 4% paraformaldehyde for 15 min at room temperature. After permeabilization and blocking (permeabilization solution: 3% bovine serum albumin fraction V, 0.3% Triton X-100, 5% horse serum in phosphate buffered saline (PBS)), cells were incubated with anti-nitrosocysteine antibody (1:50) overnight at 4 °C. After incubation with a biotin-conjugated anti-rabbit antibody (1:500), cells were labeled with streptavidin-fluorescein and visualized by fluorescence microscopy (magnification 1:40). Serving as a negative control, fixed and permeabilized cells were preincubated with 0.8% HgCl₂ for 1 h at 37 °C as described previously (12).

**Mass spectrometry analysis.**

Mass spectrometry analysis was conducted in the Hanson Institute Protein Core Facility (Adelaide, Australia). Protein samples that had been subjected to the biotin switch assay were excised from Coomassie blue-stained gels, digested with trypsin and the resulting peptides desalted into an electrospray-ionization quadrupole/time-of-flight mass spectrometer (Q-TOF2, Micromass, Manchester, UK) through a C18 reverse-phase silica column. Automated MS/MS sequencing was carried out using Data Directed Analysis (DDA) techniques. The data was then analyzed by using ProteinLynx Global Server 2.2 to determine possible sequence tags from fragmented ions and the SwissProt protein database searched for possible matches.

**Fluorescence in situ DNA binding assay for detecting NF-κB activation.**

After high glucose treatment, HUVEC were seeded onto fibronectin coated 8-well chamber slides and cultured under the same conditions overnight in the presence or absence of the ROS inhibitors. Serving a positive control for NF-κB activation, cells were stimulated with 10 ng/ml TNFα for 20 min. The cells were washed 1x with PBS then fixed with 4% paraformaldehyde. The assays were performed essentially as described in (16) with the following modifications. FITC-labeled oligodeoxynucleotides (Geneworks, Thebarton, Australia) including the NFκB binding site of the E-selectin gene (FITC-5'TTTACTGATGTCCAGGATGCCA TTGGGATTTCTCTG-3') was used to detect DNA binding activity. FITC-5’ CTTTACTGATGTCCAGGATGCCAT TGAGCTATCTCACTG-3’ was used as a negative control. Epifluorescence microscopy was performed on an Olympus BX-51 microscope equipped with excitation filters for fluorescein (494 nm), acquired to a Cool Snap FX, charge-coupled device (CCD) camera (Photometrics, Phoenix, AZ). Images were adjusted for brightness and contrast with V++ software (Digital Optics Ltd., Auckland, New Zealand).
**Statistical Analysis**

Data are expressed as mean±SEM. One-way ANOVA and unpaired Student *t* tests were used for comparison between groups. A value of *P*<0.05 was considered statistically significant.

**Results**

*High glucose reduces the S-nitrosylated protein content in HUVEC.*

To assess the effect of high glucose on protein S-nitrosylation, we incubated endothelial cells with 5.5 or 30 mmol/L glucose for 3 days, as this represents a useful model for the study of hyperglycemic injury on the endothelium (14). By using the biotin switch assay as described by Jaffrey and Snyder (15), we were able to detect significant amounts of S-nitrosylated proteins in HUVEC under normal culture conditions (Fig 1A), which is consistent with previous reports showing a considerable basal level of protein S-nitrosylation in endothelial cells (12;13). Interestingly, exposure of HUVEC to high glucose for 3 days resulted in an overall decrease in the levels of S-nitrosylated proteins, compared with the cells cultured under normal glucose conditions. Similar results were obtained when cells were cultured under high glucose conditions for up to 1 week (data not shown). The high glucose-induced reduction of protein S-nitrosylation was further confirmed by immunostaining with anti-S-nitrosocysteine antibodies as shown in Fig. 1B. However, there was no significant change in the content of S-nitrosylated proteins when cells were exposed to high glucose for less than 48 hrs (data not shown), indicating a chronic effect of high glucose. Serving as a control, HUVEC cultured with mannitol at 30 mmol/L had no effect on the protein S-nitrosylation (data not shown), ruling out a possible influence of osmotic stress.

**Effect of GSNO on high glucose-induced reduction of protein S-nitrosylation.**

The effect of hyperglycemia has been well-documented to decrease endothelial NO production or its availability (6;7). We therefore examined whether the decreased protein S-nitrosylation could be restored in a cell free system by treatment of the cell lysates with a physiological trans-nitrosylating agent, nitrosylated glutathione (GSNO). We found that incubation with the nitric oxide donor GSNO mildly increased the overall level of S-nitrosylation in cell extracts derived from either normal or high glucose-treated HUVEC (Fig. 2). Serving as a control, reduced glutathione (GSH) has no effect on protein S-nitrosylation and therefore the result reflects the level of endogenous S-nitrosylation. Interestingly, following the incubation with GSNO, the high glucose-induced reduction of S-nitrosylated proteins was not restored to the control levels (Fig 2), suggesting that the low level of nitric oxide availability is not the only cause of reduced endogenous protein S-nitrosylation under high glucose conditions.

**Characterization of high glucose-induced reduction of S-nitrosylated proteins.**

To characterize the reduced S-nitrosylated proteins by high glucose, we performed a purification of the S-nitrosylated proteins using the biotin switch method followed by proteomic analysis. Two proteins were identified by mass spectrometry analysis: GRP78 and β-actin. As numerous proteins have been reported as targets for S-nitrosylation, we
then examined the effect of high glucose on known S-nitrosylated proteins by a series of Western blot assays. Endogenous S-nitrosylated proteins were precipitated from HUVEC exposed to 5.5 or 30 mmol/L glucose for 3 days by the biotin switch method and then detected by individual immunoblotting assays. As shown in the Table 1, a total of 10 S-nitrosylated proteins were identified. 7 proteins showed significant reductions in their S-nitrosylation status following high glucose treatment.

**ROS inhibitors reverse the effect of high glucose on S-nitrosylation.**

Oxidative stress arising chiefly from ROS production is thought to underlie many of the deleterious effects of high glucose on the vasculature in diabetes (4). We therefore addressed whether the high glucose-induced overproduction of ROS is affecting the level of S-nitrosylation. To this end, we treated endothelial cells with various inhibitors of NAD(P)H oxidase (NOX) and nitric oxide synthase (NOS), which are believed to be chiefly responsible for ROS production under high glucose conditions (reviewed in (17)). As shown in figure 3A, in the presence of the NOX and NOS inhibitor DPI, high glucose-induced reduction of global protein S-nitrosylation was restored to a level approaching that observed in control cells. The same results were obtained with the NOX inhibitor apocynin, and the superoxide scavenger TEMPO (data not shown). In addition, the tricarboxylic acid cycle has been also identified as a major source of ROS induced by high glucose, as blocking of mitochondrial electron transport chain (mETC) by various inhibitors is capable of preventing the high glucose-induced ROS production (18). Interestingly, the mETC inhibitors CCCP, 4-OHCA or TTFA have similar effects to that of the NOX and/or NOS inhibitors to completely restore the decreased levels of S-nitrosylation in high glucose-treated cells (Fig 3B). Taken together, these results suggest a key role for ROS in mediating high glucose-induced reduction of protein S-nitrosylation in endothelial cells.

**Effect of high glucose on NF-κB S-nitrosylation and activation**

We and others have previously reported that high glucose was able to activate the transcription factor NF-κB (14;18), whereas NO has been shown to inhibit (19) NF-κB activation through S-nitrosylation (20;21). Therefore, we sought to examine whether high glucose-induced activation of NF-κB is due to a reduction of S-nitrosylation. Under normal culture conditions NF-κB was S-nitrosylated in HUVEC (Fig 4A). Following incubation of the cell extracts with GSNO the level of S-nitrosylation was mildly increased. By contrast, endogenous S-nitrosylation of NF-κB was barely detectable in cells exposed to high glucose. Although GSNO increased the extent of S-nitrosylation, it failed to restore NF-κB S-nitrosylation to that seen in control cells. Similar to the high glucose-induced reduction of overall protein S-nitrosylation, the decreased NF-κB S-nitrosylation was also completely reversed through treatment with the mETC inhibitors, either TTFA or CCCP or 4-OHCA (Fig 4B). Consequently, the high glucose-induced increase in NF-κB activity, as measured by a fluorescence in situ DNA binding assay, was completely inhibited by the mETC inhibitors (Fig. 4C), which is consistent with a previous report by Nishikawa, et al (18). These observations suggest that high glucose activates NF-κB by inducing ROS.
production and the resultant inhibition of NF-κB S-nitrosylation.

Discussion

In the present study, we for the first time report that high glucose was able to attenuate the overall level of protein S-nitrosylation in primary human endothelial cells. We have identified at least 7 proteins that were reduced by high glucose in their S-nitrosylation status as shown in the Fig. 1 and Table 1. These decreased S-nitrosylated proteins can be classed as (i) cytoskeleton proteins (β-actin, paxillin, vimentin and vinculin), (ii) metabolic enzymes (eNOS and diacylglycerol kinase α), (iii) chaperone (GRP78), (iv) signaling molecules (H-ras and ERK-1) and (v) transcription factor (NF-κB). It is noted that although the level of a vast majority of S-nitrosylated proteins was decreased, the extent of a number of them was actually either increased or no changed under high glucose conditions, suggesting a specific role for high glucose in the regulation of protein S-nitrosylation. As S-nitrosylation has been implicated in the regulation of numerous protein activities (10), the findings that high glucose regulates protein S-nitrosylation in endothelial cells could provide a new insight into the mechanisms underlying hyperglycemic injury on the vasculature.

Over 100 individual proteins have been identified as targets for S-nitrosylation, whilst the mechanisms underlying regulation of S-nitrosylation are still unclear. The extent of protein S-nitrosylation in endothelial cells decreases following exposure to the pro-inflammatory mediators TNF and oxLDL (12) and increases in cells under shear stress (13). TNF and oxLDL induce superoxide release in endothelial cells (5), whereas shear stress reduces oxidative stress by increasing the expression of antioxidant molecules such as superoxide dismutase and raising the intracellular levels of glutathione and NO (22). S-nitrosylation of any given target protein is thought to be influenced by the rate of formation of biologically relevant nitrosylating species, or the rate of denitrosylation (9). High glucose is known to reduce the bioavailability of NO in the cell, so the decreased S-nitrosylation may have been caused by limited availability of nitrosylating species. However, although incubation with GSNO increased the overall level of protein S-nitrosylation, the reduced S-nitrosylation in high glucose-treated cells was not restored to the level detected in the control cells following GSNO incubation in vitro. This indicated that decreased S-nitrosylation was not solely due to limiting NO availability in endothelial cells. Whether the rate of denitrosylation is enhanced by high glucose and thus accounts for the decreased protein S-nitrosylation needs further investigation.

One of the most important findings reported herein is that the inhibitory effect of high glucose on protein S-nitrosylation was completely abrogated by inhibiting ROS generation. Multiple lines of evidence have shown that the overproduction of superoxide under high glucose conditions, in particular by mitochondria that have been uncoupled by the flux of NADH from the excess glycolysis, results in an inhibition of GAPDH and subsequent accumulation of glycolysis intermediates (23). It has been reported that inhibition of ROS production prevented the high glucose-induced activation of aldose reductase and
hexosamine pathways, PKC activation and formation of advanced glycosylation end-products (18). Furthermore, treatment of streptozotin induced diabetic rats with the antioxidant N-acetylcyesteine prevented the diabetes associated defects in endothelium dependent relaxation (24). Therefore, oxidative stress has been proposed as a unifying mechanism responsible for the initiation of hyperglycemia-associated endothelial dysfunction and vascular injury. ROS can affect many signaling pathways, such as G-proteins, protein kinases, ion channels, and transcription factors including NF-κB (25). Enhanced oxidative stress can also interfere with the availability of NO.

We found that the high glucose-induced reduction of protein S-nitrosylation was reversed in the cells treated with either non-specific antioxidants, including apocynin, DPI and the superoxide scavenger TEMPOL, or with the mETC specific inhibitors TTFA, CCCP or 4-OHCA that have been previously shown to completely block ROS formation in endothelial cells under high glucose conditions (18). Although to some degree variations were observed amongst these different ROS inhibitors (Fig. 3 and Fig. 4), they are able to profoundly prevent high glucose induced reduction of protein S-nitrosylation, suggesting a causal role for ROS. It has been recently demonstrated that superoxide was able to induce decomposition of S-nitrosothiols in a dose- and time-dependent manner (26). In addition, amongst the various S-nitrosylated proteins, the majority are reportedly regulated at a single critical cysteine residue within an acid-base or hydrophobic structural motif, which could also be subject to oxygen- or glutathione-dependent modification (9), suggesting a possible role for superoxide in the regulation of S-nitrosylation. On the other hand, NO has also been suggested to regulate the activity of thioredoxin, a key endogenous antioxidant, through S-nitrosylation on Cys 69, representing a counterpoising connection between these two important gaseous signalling pathways. Interestingly, Schulze et al (27) reported that NO also suppresses mRNA expression of the thioredoxin inhibitory protein Txnip in vascular smooth muscle cells via a NO-responsive cis-regulatory element in the Txnip promoter, an effect that is abrogated by high glucose, suggesting an additional molecular mechanism whereby high glucose induces endothelial dysfunction.

NF-κB is a master transcription factor that regulates a number of genes that are critically involved in the regulation of endothelial function as well as the pathogenesis of various vascular disorders. Hyperglycemia has been reported to activate NF-κB through activation of protein kinase C (19) and the sphingosine kinase (14) pathways, or via overproduction of ROS (18). By contrast, NO is a potent inhibitor of NF-κB activation (28). Interestingly, inhibitory κB kinase β (IKKβ), the catalytic subunit required for the activation of NF-κB, has been demonstrated as a direct target of S-nitrosylation resulting in loss of IKKβ activity (21). Additionally, the p50 subunit of NF-κB has been also reported to be S-nitrosylated, causing an inhibition of its binding to DNA (20). Thus, NO is capable of acting at multiple points along the pathway to attenuate NF-κB signaling through S-nitrosylation. We found that the NF-κB S-nitrosylation was significantly decreased in the high glucose-treated endothelial cells and that this reduction was completely reversed by inhibition of
ROS generation. Consequently, the high glucose-induced inhibition of NF-κB activity was blocked by the ROS inhibitors (Fig. 4). These observations suggest that the decreased S-nitrosylation of NF-κB may play a role in the oxidative stress-mediated NF-κB activation in endothelial cells under high glucose conditions.

In conclusion, we have shown that high glucose dramatically alters the global pattern of protein S-nitrosylation along with a significant reduction of the S-nitrosylated proteins in endothelial cells. The effect of high glucose was prevented by ROS inhibitors, suggesting that oxidative stress may either prevent S-nitrosylation or accelerate denitrosylation. Although the direct functional consequences of such alteration in S-nitrosylation of specific proteins remain to be identified, the changes in S-nitrosylation may contribute to hyperglycemia-induced endothelial dysfunction and vascular lesions. This finding could pave a new way to designing new strategies for prevention and treatment of diabetes-associated cardiovascular diseases.

Acknowledgments

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Reference List


Table 1. Identification of S-nitrosylated proteins in high glucose-treated cells.

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Cell lysates derived from HUVEC that were exposed to NG or HG for 3 days were subjected to the biotin switch assay as described in the ‘Methods’. Precipitated S-nitrosylated proteins were separated by SDS-PAGE and then identified by mass spectrometry analysis (MS/MS) or Western blot assays (WB). *Not previously reported. ↓, the level of S-nitrosylated protein was decreased in cells exposed to high glucose; and –, no changes, in comparison with the controls; n, number of independent experiments.
Figure Legends

Figure 1. Effect of high glucose on protein S-nitrosylation in HUVEC. HUVEC were exposed to 5.5 (NG) or 30 mmol/L glucose (HG) for 3 days. (A) Whole cell lysates were subjected to the biotin switch assay as described in the ‘Methods’. S-nitrosylated proteins were separated by SDS-PAGE and immunoblotted with anti-biotin antibodies. The bottom immunoblot was conducted with whole cell lysates and probed with anti-vinculin antibodies, showing an equal level of protein loading. (B) Treated cells were fixed and immunostained with anti-nitrosocysteine antibodies as described under ‘Methods’. Data are representative of similar results in at least three separate experiments with different primary HUVEC lines.

Figure 2. Effect of GSNO on protein S-nitrosylation. Lysates from HUVEC treated as described in Fig. 1, were incubated with 100 µM GSNO or GSH for 20 min at room temperature in the dark, and then subjected to the biotin switch assay. S-nitrosylated proteins were separated by SDS-PAGE and immunoblotted with anti-biotin antibodies. The bottom immunoblot of whole cell lysates was blotted with anti-actin antibodies, showing an equal level of proteins loading. A representative Western blot is shown from at least three separate experiments with different primary HUVEC lines.

Figure 3. Effect of ROS inhibitor on S-nitrosylation in HUVEC. (A) and (B) After a 3-day incubation with NG or HG and treatment with (A) 10µM diphenyleneiodonium (DPI) or (B) 500 nM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 10 µM thenoyltrifluoroacetone (TTFA) or alpha-cyano-4-hydroxycinnamic acid (4-OHCA) for 16 hrs, cell lysates were then subjected to the biotin switch assay and immunoblot analysis with anti-biotin antibodies. Anti-vimentin antibodies were used to control for loading. Data are representative of similar results in at least three separate experiments with different primary HUVEC lines.

Figure 4. Effect of high glucose on S-nitrosylation and activation of NFκB. (A) Cell lysates derived from HUVEC exposed to NG or HG for 3 days were incubated with GSH or GSNO and then subjected to the biotin switch assay as described in the ‘Methods’. Streptavidin-agarose purified S-nitrosylated proteins, along with 20 µl of the lysates, were separated by SDS-PAGE and immunoblotted with anti-NF-κB antibodies. The equal expression levels of NF-κB were shown in the immunoblot of the original lysates (left two lanes). (B) HUVEC were incubated with NG or HG for 3 days and treated with 500 nM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 10 µM thenoyltrifluoroacetone (TTFA) or alpha-cyano-4-hydroxycinnamic acid (4-OHCA) for 16 hrs, the level of S-nitrosylated NF-κB was then determined by the biotin switch assay. Results are representative of three independent experiments on different primary cell lines of HUVEC. A combined densitometric analysis is shown in the lower panel. * P < 0.05, HG vs NG; ** p<0.05, ROS inhibitor-treated vs HG alone. (C) NG or HG-treated cells were treated with CCCP or TTFA, NF-κB activity was then measured by fluorescence in situ DNA binding assay as described in the ‘Methods’. The NFκB reactive oligonucleotide fluorescence was not found in HUVEC cultured with NG, whereas the fluorescence accumulated in the nuclei of cells exposed to HG and detected
by epifluorescence microscopy. Phase-contrast micrographs are shown in the left panel. Data are representative of similar results in at least three separate experiments with different primary HUVEC lines.
Figure 1.

A. 

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B. 

NG

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Biotin

Actin
Figure 3.

A.

B.
Figure 4.

A. Lysates vs. Pull-downs

B. Streptavidin Pulldown

C. Phase vs. FITC