Nitric Oxide Directly Promotes Vascular Endothelial Insulin Transport

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Running title: Nitric oxide promotes endothelial insulin transport

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Word count: 4,900
Number of figures: 8
Abstract

Insulin resistance strongly associates with decreased nitric oxide (NO) bioavailability and endothelial dysfunction. In the vasculature NO mediates multiple processes affecting insulin delivery, including dilating both resistance and terminal arterioles in skeletal muscle in vivo. However, whether NO directly regulates vascular endothelial cell (EC) insulin uptake and its trans-endothelial transport (TET) is unknown. Here we report that L-NAME pre-treatment blocked while L-arginine or sodium nitroprusside (SNP), each enhanced EC uptake of fluoroisothiocyanate (FITC)-labeled insulin. SNP also partly or fully reversed the inhibition of EC insulin uptake caused by L-NAME, wortmannin, the Src inhibitor PP1 or TNFα. In addition, SNP promoted [125I]TyrA14-insulin transendothelial transport by ~40%. Treatment with insulin ± SNP did not affect EC cGMP levels, and the cGMP analogue 8-bromo-cGMP did not affect FITC-insulin uptake. In contrast, treatment with insulin and SNP significantly increased EC protein S-nitrosylation, the co-localization of S-NO and protein-tyrosine phosphatase 1B (PTP1B) and Akt phosphorylation at Ser473 and inhibited PTP1B activity. Moreover, a high-fat diet significantly inhibited EC insulin-stimulated Akt phosphorylation and FITC-insulin uptake that was partially reversed by SNP in rats. Finally, inhibition of S-nitrosylation by knock-down of thioredoxin-interacting protein completely eliminated SNP-enhanced FITC-insulin uptake. We conclude that NO directly promotes EC insulin transport by enhancing protein S-nitrosylation. NO also inhibits PTP1B activity thereby enhancing insulin signaling.
Key words: Nitric oxide, S-nitrosylation, insulin uptake, Insulin signaling, vascular endothelial cells

Before insulin can act on myocytes, it must first traverse the continuous vascular endothelium in skeletal muscle. Insulin delivery to muscle is affected by blood flow (1), flow distribution (2) and by trans-endothelial insulin transport (TET) (3;4). Importantly, insulin delivery to muscle interstitial fluid is a rate-limiting step in insulin’s peripheral action (5;6) and is delayed in insulin-resistant, obese humans suggesting a significant role for this transport process in peripheral insulin resistance (7;8). Endothelial dysfunction, secondary to reduced nitric oxide (NO) bioavailability, is an early and prominent feature of insulin resistance. Endothelial nitric oxide synthase (NOS-3 or eNOS) produces NO from L-arginine and eNOS is activated by insulin at physiologic concentrations. Knock-out of eNOS or inhibiting insulin signaling by endothelium-specific knock out of IRS-2 leading to the reduction of eNOS activity in the vascular endothelial cell produces metabolic insulin resistance (9;10). In addition, endothelial specific knockout of IRS-2 inhibits insulin-induced microvascular recruitment and reduces insulin delivery to muscle interstitium. However, it is not known whether the reduced insulin delivery is due to reduced blood flow, altered flow distribution, impaired trans-endothelial insulin transport or a combination of these (10). We and others have previously shown that insulin induces vasodilation by enhancing NO production to facilitate its own delivery to the peripheral tissues in vivo (1;2). Whether NO directly affects insulin uptake and TET has not been examined.
The insulin receptor and caveolae mediate EC insulin uptake (4;11-13) and this process is blunted by either inhibiting intracellular insulin signaling or treatment with TNFα. Conversely, stimulating intracellular insulin signaling by inhibiting protein-tyrosine phosphatase 1B (PTP1B) enhances insulin uptake (12).

In the present study, we found that exogenously delivered NO stimulated both the uptake and trans-endothelial transport of insulin by aortic endothelial cells. We also found that NO partially or fully restored insulin uptake by the cells pre-treated with inhibitors of insulin signaling pathways (12). To explain this we examined pathways downstream of NO production by which the NO might act on insulin uptake. We found that exogenously delivered NO can directly promote insulin transport independent of eNOS activity through enhancing protein S-nitrosylation, including that of PTP1B without affecting the sGC-cGMP pathway and overcome the impaired insulin transport seen with experimental insulin resistance.

RESEARCH DESIGN AND METHODS

Cell Culture
Bovine aorta endothelial cells (bAECs) (BioWhittaker, Inc., Walkersville, MD) (passage numbers 2-8) were grown in EGMMV medium.

Measurement of transendothelial insulin transport
These experiments were performed as previously described (4;14). Briefly, bAECs were seeded onto the Transwell inserts (6.5 mm diameter, 0.4 μm pore size, polyester
membrane, Corning Inc, Corning, NY) treated with human fibronectin (Sigma-Aldrich, St Louis, Missouri). The trans-endothelial electrical resistances (TEERs) were monitored daily using an Epithelial Volt-ohmmeter (EVOM, WPI, Sarasota, FL) and EndOhm chamber (EndOhm, WPI). After the TEER reached a plateau, the endothelial monolayers were washed twice at 37°C with basal medium (EBM), the fluid in the top chamber was replaced with EBM containing $^{[125]}$I$\text{Tyr}^{A14}$ insulin (200 pmol/L) (PerkinElmer, Boston, MA) with or without 0.3 µmol/L SNP (Calbiochem). At selected times 200 µl of fluid was removed from the bottom chamber and this was replaced with 200 µl EBM to assure hydrostatic balance. The concentration of $^{[125]}$I$\text{Tyr}^{A14}$ insulin was measured using a gamma counter. The percentage of insulin transported was calculated.

**Animals**

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed in an animal room maintained at ~22 °C with a 12-h light/dark cycle and fed ad libitum. Rats were randomly assigned to either an high fat diet (HFD) (60% fat, 20% protein, and 20% carbohydrate; product # D12492, Research Diets, New Brunswick, NJ) or a control regular chow diet (CHOW) (product # 7012; Harlan Laboratories) for 4 weeks. Rats were euthanized by CO$_2$. The aorta was quickly dissected and put into 5% CO$_2$ in O$_2$-bubbled modified Krebs buffer (NaCl 119 mmol/L, KCl 4.7 mmol/L, CaCl$_2$·2H$_2$O 2.5 mmol/L, MgSO$_4$7H$_2$O 1.17 mmol/L, NaHCO$_3$ 25 mmol/L, KH$_2$PO$_4$ 1.18 mmol/L, EDTA 0.027 mmol/L, glucose 5.5 mmol/L). The surrounding fascia was carefully removed and the vessel was then divided into small segments and cut open. After stabilization in a cell culture incubator for 1 hour, these aortic segments were
treated with 0.3 or 30 µmol/L SNP with or without 50 nmol/L FITC-insulin (Sigma-Aldrich, St Louis, Missouri) or regular insulin for 30 min. After fixation with cold methanol, the endothelial face of the vessel was placed face down onto a coverslip that was pre-coated with poly-l-lysine (Sigma-Aldrich, St Louis, Missouri), pressure was applied briefly, the vessel wall was removed and the coverslip with adherent ECs was processed for immunocytochemical staining (see below). The study procedure was approved by the animal care and use committee of the University of Virginia.

**Western Blotting**

Western blotting was performed as described previously(13;15). Briefly, after blocking with 5% low-fat milk in Tris-buffered saline (TBS) plus Tween 20, membranes were incubated overnight at 4°C with monoclonal antibody against thioredoxin-interacting protein (Txnip) (MBL International Corporation, Woburn, MA) or polyclonal antibody against caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA), or monoclonal antibody against GAPDH (Sigma-Aldrich, St Louis, Missouri), respectively. This was followed by incubation with a species-specific secondary antibody coupled to horseradish peroxidase (Amersham Life Sciences, Piscataway, NJ), and the blots were developed using an enhanced chemiluminescence Western blotting kit (Amersham Life Sciences, Piscataway, NJ). The developed films were scanned using a densitometer (Molecular Dynamics, Piscataway, NJ) and quantified using the ImageQuant 5.0 software.

**Real time reverse- transcription PCR**
Real time RT-PCR assay was performed as described previously(13;14). Briefly, total RNAs were extracted from the cultured bAECs with RNeasy kit (Qiagen) and were reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). The cDNA products were then amplified using the iQ SYBR green supermix and the iCycler apparatus (Bio-Rad). For the amplification of Txnip gene products, the following primers were designed: forward 5’-CATGTGGAGGAGGAGCAATTTA-3’ and reverse 5’-GCCAGTTACTACTGCTTTATG-3’, and the Txnip mRNA levels were normalized to the housekeeping gene (GAPDH) mRNAs (primers designed: forward 5’-GGGCATCATCTCTGCACCT-3’, and reverse 5’-GGTCATAAGTCCCTTCACAG-3’)(Integrated DNA Technologies, Coralville, IA). Standard curves for each mRNA were generated by serial dilution of cDNA synthesized from the extracted total RNA and was included in each iCycler real-time PCR experiment. The specificity of the desired product was verified by the analysis of the melting curve.

**siRNA Design and Transfection**

A specific siRNA duplex against bovine Txnip mRNA and a scrambled siRNA control were purchased from Dharmacon, Inc. (Lafayette, CO). Cells were seeded and transfected when they reached 30~50% confluence with siRNA duplex to a final concentration of 40 nmol/L using Oligofectamine (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection cells were serum-starved for 6 hours followed by insulin treatment as described previously (13;14).

**Immunocytochemistry**
The double-staining protocols were same as described previously (4;12). Briefly, the methanol-fixed bAECs were washed three times in TBS, permeabilized in TBS containing 0.05% Triton X-100 and 1% horse or goat serum for 30 min at room temperature, and incubated with two different primary antibodies against two different target proteins (double labeling) overnight at 4°C. The following primary antibodies were used: rabbit polyclonal anti-FITC (Molecular Probes, Inc., Eugene, OR), mouse monoclonal anti-Txnip (MBL International Corporation, Woburn, MA), monoclonal anti-PTP1B (Abcam Inc, Cambridge, MA) and monoclonal anti-phospho-Akt (Ser473) (Millipore). The cells were washed three times in TBS, and then incubated with species-specific secondary antibodies conjugated with a fluorochrome Cy2 or Cy3 (Jackson ImmunoResearch, West Grove, PA) at 1:200 dilutions for 45 min at room temperature. The cells were washed three times in TBS, and then cover-slipped with the anti-fade mounting medium with DAPI.

**Assay for Cyclic Guanosine Monophosphate (cGMP)**

Intracellular cGMP concentration of the cultured bAECs was measured using the cGMP EIA kit (Cayman Chemical). The sensitivity of the assay was enhanced by the optional acetylation procedure according to the manufacturer’s protocol. Cells were treated with or without isobutylmethylxanthine (IBMX, 200µmol/L) (Sigma-Aldrich, St Louis, Missouri) for 10 min before insulin and/or SNP stimulation. Protein concentration was measured by Bradford method. The measured cGMP values were normalized against the corresponding protein concentrations.
**Rat’s blood analyses**

Arterial serum glucose concentrations were measured using Cayman’s Glucose Colorimetric Assay Kit (Cayman Chemical). Serum insulin (Mercodia AB, Uppsala, Sweden) and triglycerides (TG) (Cayman Chemical) concentrations were measured using ELISA assays.

**In Situ Detection of PTP1B S-nitrosylation**

The general protein S-nitrosylation in the ECs harvested from rat aortas (see above) was evaluated in situ using “Biotin-switch” technology-based S-Nitrosylated Protein Detection Assay Kit (Cayman Chemical) according to the manufacturer’s protocol. The biotin derivatization was detected by the included fluorescein-conjugated avidin. In these experiments assay of the protein S-nitrosylation was determined in combination with the immunocytochemical staining for PTP1B using the monoclonal primary antibody against PTP1B followed by a specie-specific secondary antibody conjugated with Cy3 and visualized by confocal imaging.

**PTP1B Enzymatic Activity Assay**

The PTP1B activity of bAECs was measured after precipitation of PTP1B using a modified immunoprecipitation procedure described previously (15;16). Briefly, cultured bAECs were scraped in ice-cold lysis buffer (PBS containing 1% NP-40, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L PMSF, and 0.1 mmol/L leupeptin). The lysates were gently rocked at 4°C for 15 min, and then centrifuged at 14000xg for 10 min at 4°C. The supernatants were pre-cleared by adding 20 µl/ml of cell lysate Protein A/G Plus Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for
10 min. Equal amounts of protein samples (500 µg of total protein) were immunoprecipitated with the monoclonal anti-PTP1B antibody at 4°C overnight. PTP1B immunocomplexes were further precipitated by the Protein A/G Plus Agarose at 4°C for an additional 2 h. Immunoprecipitates were washed 5 times with TBS and the residue TBS buffer was removed. The activity of purified PTP1B was assayed using the PTP1B Assay kit (Calbiochem) according to the manufacturer’s protocol. Briefly, the phosphopeptide substrate (IR5, containing a sequence from the insulin receptor β subunit domain that must be autophosphorylated to achieve full receptor kinase activation) were added to a final concentration of 75 µmol/L in a total reaction volume of 100 µl in the assay buffer. The sample mixtures were incubated for 30 min at 30°C. After the reaction, 60 µl aliquots were placed into half area 96-well plates, and 25 µl of Red Reagent plus 40 µl assay buffer were added into each sample well and gently mixed. After incubation at room temperature for 30 min, the absorbance was measured at 620 nm using a plate reader.

**Imaging**

The immunocytochemical labeling was examined using a confocal microscope as described previously (4;12-15). Confocal imaging used a Leica SP5 X imaging system equipped with UV(405 nm), tunable (470-670 nm) white light and Argon-ion lasers (458, 477, 488, 496, 514 nm). 40× and 60× 1.4 NA oil-immersion lenses were used to acquire optical sections. During image acquisition the individual microscopic field was selected to include similar number of cells but was otherwise random. To quantify fluorescence intensity, the images from randomly selected microscopic fields containing similar
number of nuclei staining were outlined and the integrated fluorescence intensities were measured using the Image J software. In the case (figure 5A) that only a few cells were included in each microscopic field as the protocol for the S-Nitrosylated Protein Detection Assay Kit requires use of 4% paraformaldehyde with the specific wash buffer for cell fixation which appeared to inhibit intact rat ECs attachment to the cover-slip, individual cells were outlined by polygonal method (30 cells for each group were randomly selected) and the integrated fluorescence intensities were measured. Digital images were processed identically with Adobe Photoshop.

**Statistical Analysis**

Data are presented as mean ± SEM. Statistical comparisons among different groups were made using One Way ANOVA with Student-Newman-Keuls post-hoc testing. Statistical significance is defined as p ≤ 0.05.

**Results**

**Nitric oxide production regulates FITC-insulin uptake**

We first examined the effect of NG-nitro-L-arginine methyl ester (L-NAME) inhibition of nitric oxide synthase on FITC-insulin uptake. Figure 1A shows that compared to control, pre-treating bAECs with L-NAME strongly inhibited FITC-insulin uptake (p<0.05). Conversely, pretreatment of bAECs with 500 µmol/L L-arginine (the substrate of eNOS) for 30 min significantly increased FITC-insulin uptake (Fig. 1B and 1D),
whereas pretreatment of cells with D-arginine had no effect. L-NAME added with L-arginine blocked the increased uptake seen with L-arginine alone (Fig. 1B and 1D). We then examined whether L-arginine’s effect on FITC-insulin uptake could be mimicked by giving NO to bAECs. Adding modest concentrations (0.01~0.3 µmol/L) of sodium nitroprusside (SNP), a NO donor, significantly increased FITC-insulin uptake by bAECs compared to FITC-insulin alone (p<0.05) (Fig. 1C and 1D) (also see Fig. 6F and 6H). However, SNP at higher concentrations did not stimulate insulin uptake (Fig. 1C) suggesting a biphasic action with an inhibitory effect of higher SNP (NO) concentrations (17-19). Interestingly, 0.3 µmol/L SNP also eliminated the inhibitory effect of L-NAME on FITC-insulin uptake (Figure 1E; also see 1A).

Next, we examined the effect of SNP on $[^{125}\text{I}]\text{Tyr}^{14}\text{insulin}$ ($^{125}\text{I}$-insulin) transendothelial transport using a Transwell device (4;14). Figure 2 shows that compared to the control, adding SNP increased $^{125}\text{I}$-insulin transendothelial transport by ~40% at both 10 and 60 min, respectively (p<0.05, for each time point). In aggregate, these data suggest that the nitric oxide donor, SNP may directly promote insulin transport in an eNOS activity-independent fashion.

**Nitric oxide rescues the inhibition of insulin uptake induced by blocking intracellular insulin signaling pathways**

We previously reported that insulin transport by bAECs is dependent on its intracellular insulin signaling as either general inhibition of tyrosine kinases (genistein) or more specific inhibition of Src (PP1), phosphatidylinositol-3 (PI3K) (wortmannin) or mitogen-
activated protein kinases (MAPK) (PD 098059) (12) each inhibited FITC-insulin uptake. Therefore, we tested whether adding SNP to bAECs relieved the inhibition of FITC-insulin uptake induced by blocking these intracellular insulin signaling pathways. Figure 3 and Supplemental figure 1 confirm that pretreatment of bAECs with wortmannin, genistein, PP1 or PD 098059 significantly inhibited FITC-insulin uptake as reported previously (12). Adding SNP however completely rescued both wortmannin- and PP1-inhibited insulin uptake (Fig. 3A and 3B; Fig. 3C) and partially restored FITC-insulin uptake that had been inhibited by PD 098059 (Supplemental figure 1B). SNP did not significantly affect genistein-inhibited FITC-insulin uptake (Supplemental figure 1A). These data indicate that NO is able to promote FITC-insulin uptake despite pre-inhibiting some intracellular insulin signaling pathways.

We have previously used TNFα treatment of ECs as an in vitro model of insulin resistance and shown that treatment of bAECs with TNFα inhibited FITC-insulin uptake (12). Figure 3D shows that adding 0.3 µmol/L SNP not only completely rescued TNFα-induced inhibition of insulin uptake but strikingly stimulated FITC-insulin uptake.

**SNP enhanced insulin transport requires protein S-nitrosylation but not activation of soluble guanylyl cyclase**

We next examined the pathways by which SNP promoted FITC-insulin transport. The selective, irreversible, heme-site inhibitor of soluble guanylyl cyclase (sGC), ODQ (20), completely eliminated the enhanced FITC-insulin uptake induced by SNP compared to the vehicle control (Fig. 4A and 4C). However, the membrane permeable cGMP
analogue 8-bromo-cGMP over a range of concentrations had no significant effects on FITC-insulin uptake (Fig. 4B). In addition, treatment of ECs with insulin ± SNP did not significantly affect the intracellular cGMP levels (Supplemental figure 2), consistent with a previous report (21). This seeming inconsistency may be explained by the observation that to exert its biological effects as a NO donor, higher concentrations of SNP (>30 nmol/L) require intracellular bioactivation (metabolic NO formation) that is susceptible to inhibition by ODQ (22). In aggregate, these data suggest that SNP-enhanced insulin transport may not be mediated by activation of soluble guanylyl cyclase.

Since NO also regulates cellular function by S-nitrosylation, we next examined the effect of SNP treatment on protein S-nitrosylation. Figure 5A (top row) and 5B show that both insulin and SNP treatment increased the protein S-nitrosylation level, and SNP plus insulin treatment further significantly increased the protein S-nitrosylation compared to either insulin or SNP alone in the intact aortic endothelial cells of rats ex vivo. Since the steady-state level of protein S-nitrosylation is determined by the balance between nitrosylation and denitrosylation, thioredoxin activity (a broad spectrum denitrosylase) is important for determining the steady state levels of protein S-nitrosylation (23). Txnip has recently been demonstrated to critically inhibit protein denitrosylation (19). In order to examine the effect of reducing S-nitrosylation on SNP-stimulated FITC-insulin uptake, we designed a specific siRNA against Txnip in bAECs to silence Txnip expression and thereby promote protein denitrosylation. Figure 6A and 6B show that compared to the scrambled siRNA, the siRNA against Txnip reduced Txnip protein expression by ~75%. Neither caveolin-1 nor GAPDH protein expression was affected by knockdown of Txnip.
Figure 6C-H show that insulin significantly increased Txnip staining compared to the basal medium control (p<0.05), and 0.3 µmol/L SNP plus insulin further increased Txnip staining compared to insulin alone (p<0.05) (Fig. 6C and 6E; 6F and 6G, also see 6I) both in vitro and ex vivo, though higher SNP dose (30 µM) had no additional effect on Txnip staining (Fig. 6F and 6G, also see 6I). Interestingly, the increased Txnip staining induced by insulin or insulin plus 0.3 µmol/L SNP was paralleled by enhanced protein S-nitrosylation (Fig. 5A upper row and 5B). On the other hand, insulin with or without 0.3 µmol/L SNP treatment did not affect Txnip mRNA expression (p>0.05) (Fig. 6J). This suggests that these treatments inhibit the rapid turnover of Txnip proteins (19) leading to an enhanced protein S-nitrosylation (19). Additionally, compared to the scrambled siRNA control, knockdown of Txnip with the specific siRNA against Txnip (Fig. 6C right panel and 6E) strikingly reduced SNP-promoted FITC-insulin uptake (Fig. 6C left panel and 6D) proportionate to the reduced level of Txnip found (Fig. 6C right panel and 6E). These data indicate that NO may have promoted insulin transport not through activation of soluble guanylyl cyclase but through promotion of S-nitrosylation of proteins.

Since we previously reported that inhibition of PTP1B using a specific PTP1B inhibitor significantly enhanced FITC-insulin uptake by aortic endothelial cells (12), we next examined whether treatment of aortic endothelial cells with insulin and/or 0.3 µmol/L SNP affected the S-nitrosylation of PTP1B and its enzymatic activity. Figure 5A shows that although 0.3 µmol/L SNP only or insulin only treatment obviously increased PTP1B protein S-nitrosylation, 0.3 µmol/L SNP plus insulin treatment caused the most robust
PTP1B protein S-nitrosylation as indicated by almost complete co-localization of S-NO and PTP1B (Fig. 5A bottom row) compared to the basal medium control. In addition, vehicle plus insulin treatment tended to inhibit PTP1B activity but it was not statistically significant; however, 0.3 µ mol/L SNP plus insulin treatment almost completely inhibited PTP1B activity (p<0.05) compared to either basal medium or vehicle plus insulin controls (Fig. 5C). These data suggest that NO may inhibit PTP1B activity via S-nitrosylation of PTP1B protein to regulate insulin transport.

**Nitric oxide stimulates intracellular insulin signaling and reverses HFD-induced impairments in insulin signaling and uptake in Sprague-Dawley rats**

Finally, we examined the effects of NO on intracellular insulin signaling by in situ detection of changes in Akt phosphorylation at Ser^473^ using fresh rat aortic ECs attached to cover slips. Since high fat feeding of rats for 3–4 weeks has been shown to cause whole body insulin resistance and vascular dysfunction (24;25), we also examined the effects of feeding rats a HFD for 4 weeks on vascular endothelial insulin signaling and uptake and responsiveness to SNP. Supplemental figure 3A-D show that although serum glucose concentrations were not changed, body weight, serum TG and insulin were increased by HFD feeding consistent with a state of insulin resistance as previously reported (24;25). In the rats on the regular chow diet, 0.3µ mol/L SNP significantly increased insulin-stimulated Akt phosphorylation at Ser^473^ and FITC-insulin uptake (Fig. 7). In addition, 4 weeks-HFD feeding inhibited both FITC-insulin uptake and insulin-stimulated Akt phosphorylation at Ser^473^, and 0.3µ mol/L SNP treatment partially but significantly reversed this both effects (Fig. 7). These data combined with the data
shown in Figure 5 suggest that NO may enhance EC insulin signaling and uptake in part through inhibition of PTP1B activity (Figure 8).

Discussion

Our results provide the first demonstration that nitric oxide can directly promote both insulin uptake and trans-endothelial transport by arterial endothelial cells. Furthermore, nitric oxide (SNP, a NO donor) can completely or partially restore insulin uptake that had been inhibited by agents that interfere with signaling through PI3, Src or MAP kinases as well as by TNFα and by high fat feeding in vivo. SNP did not reverse the inhibition of insulin uptake provoked by genistein, a nonspecific tyrosine kinase inhibitor. Although NO exerts its diverse cellular actions via enhancing both sGC-cGMP-protein kinase G and protein S-nitrosylation-mediated signaling pathways, the data presented here supports an important role for the latter pathway in mediating nitric oxide-stimulated insulin uptake and transport. Taken together, our current results indicate a third role for NO related to facilitating insulin delivery to muscle tissue. This action, coupled with its vasodilatory actions to increase muscle blood flow (1) and improve flow distribution (2;26), suggests a highly coordinated physiological role for NO to promote insulin delivery.

In the present study, we found that pre-treatment of bAECs with L-NAME (100 µmol/L) completely inhibited FITC-insulin uptake, and that SNP can overcome the inhibition induced by L-NAME. This suggests that the exogenous NO from SNP is enough to enhance insulin uptake regardless of endogenous NO production. Insulin clamp studies
have shown that the eNOS -/- mouse is metabolically insulin resistant (9) and a recent in vivo study has shown that these mice manifest increased inflammation and impaired insulin signaling in aortic tissues (27).

Previous in vivo studies have shown that insulin delivery from the plasma to the interstitial fluid compartment of skeletal muscle is a rate-limiting step in insulin’s peripheral action (5;6). This process is delayed in insulin-resistant, obese subjects in whom a significantly lower interstitial insulin level is seen in the early phase of insulin infusion compared to normal controls (7;8;26). Whether the delayed interstitial delivery of insulin in insulin-resistant, obese subjects is due to a defective vasodilating response, a lower capillary density (8;26;28;29) or to a defective trans-endothelial transport (3;4;11) or a combination of these is uncertain. To dissect NO’s effects specifically on trans-endothelial insulin transport we employed a Transwell device on which a confluent bAEC monolayer mimics the endothelial boundary in the vasculature of peripheral tissues such as muscle and adipose tissues. Previous in vitro studies by us and others have used this approach and demonstrated that trans-endothelial insulin transport is receptor-mediated (3;4) and involves caveolae (11;13). This receptor-mediated process is regulated by intracellular insulin signaling (12;14). In the present study, we observed that NO acts directly on vascular endothelial cells to promote insulin’s uptake and trans-endothelial transport.

Endothelial dysfunction, characterized by a deficiency of bio-available NO, has been found to precede the development of type 2 diabetes and is significantly correlated with
insulin resistance (30). Endothelial NO production has been shown positively related to peripheral insulin sensitivity (31). Insulin itself is an important eNOS activator (32). Inhibiting insulin signaling inhibits both insulin uptake/trans-endothelial transport in vitro (12;14) and insulin trans-capillary transport in vivo (26). In the present study, we observed that SNP partially or completely relieved the inhibition of insulin uptake provoked by wortmannin, PD 98059 or PP1 but not that caused by genistein. A recent study has reported that a NO donor can directly activate PI3-K and MAPK signaling pathways in both human and rat vascular smooth muscle cells (33). On the other hand, TNFα decreases EC NO bioavailability (34) by inhibiting eNOS expression and interfering with early events in insulin signaling (35;36) and TNFα also inhibits insulin uptake by bovine aortic endothelial cells (12). Here we observed that SNP eliminated the TNFα inhibition of insulin uptake and even enhanced insulin uptake beyond that seen in control cells (Fig. 3D and E). The NO donor DETANO was reported recently to rescue the palmitate-induced inhibition of insulin-stimulated IRS-1, Akt and eNOS phosphorylation in HUVECs, whereas knockout of eNOS in vivo increased the vascular inflammation and insulin resistance (27).

The rat on HFD for 4 weeks provides a well-characterized insulin resistance model. HFD feeding for 4 weeks induces vascular insulin resistance characterized by loss of insulin-mediated microvascular perfusion in skeletal muscle, and whole body and muscle metabolic insulin resistance (24;25). Interestingly, HFD-fed mice demonstrate impaired intracellular insulin signaling in aortic tissue much earlier (within one week of HFD feeding) than that in liver, muscle and adipose tissues (37). In the present study we showed that both insulin-stimulated Akt phosphorylation and insulin uptake were
suppressed in aortic ECs from the rats on HFD for 4 weeks (Fig. 7). Treatment with 0.3 µmol/L SNP could inhibit PTP1B activity (Fig. 5A and 5C) and enhance intracellular insulin signaling and partially reverse the HFD-induced impairments in both insulin signaling and uptake.

Nitric oxide regulation of cellular function is complex (38;39) and appears to involve at least two major mechanisms under physiological conditions: 1) activation of soluble guanylyl cyclase (sGC) (38); and 2) reversible post-translational modification of proteins by S-nitrosylation or for some proteins, S-nitrosylation could be an intermediate step leading to the glutathionylation that also regulates protein function (40)). S-nitrosylation has emerged as an important feature of NO signaling (39). Indeed, we observed that SNP treatment significantly increased in situ protein S-nitrosylation in intact aortic endothelial cells ex vivo (Fig. 5). In addition, in the present study, we observed that the intracellular cGMP levels in cultured bAECs were barely detectable even after pre-inhibition of the intracellular cGMP phosphodiesterase using IBMX. Furthermore, cGMP levels were not changed after the stimulation with either insulin or SNP or both, consistent with findings reported by others (21). These results combined with the failure of 8-bromo-cGMP to stimulate insulin uptake suggest that in vascular endothelial cells enhanced cGMP generation is not necessary for SNP (or NO) stimulated insulin uptake. This led us to explore whether SNP-stimulated insulin uptake might be acting via protein S-nitrosylation. To address this we took advantage of the fact that Txnip inhibits thioredoxin (a broad spectrum denitrosylase) and cellular Txnip protein turnover is very fast (half-life 10-20 min) (19). SiRNA directed against Txnip would, by decreasing
Txnip, be expected to enhance thioredoxin-mediated reduction of protein S-nitrosylation and in the current context to diminish SNP-stimulated insulin uptake. Interestingly, in contrast to the results observed previously with either high concentrations of NO donor (≥10 μM GSNO) (18) or endogenously produced NO through the activation of iNOS induced by cytokine stimulation (19) in which the higher concentration of NO actually inhibited Txnip expression, in the current study we observed that adding insulin modestly, but significantly (p<0.05) increased Txnip staining (i.e. enhanced protein S-nitrosylation) and adding low concentrations of SNP with insulin was able to further enhance Txnip staining (Fig. 6C right panel, 6E, 6F top row and 6G, also see 6I and Fig. 5A and 5B) and correspondingly to significantly increase insulin uptake (Fig. 6C left panel, 6D, 6F middle row and 6H). In addition, the mRNA of Txnip expression was not affected by these treatments (Fig. 6J) suggesting that these treatments may have attenuated the rapid turnover of Txnip (19). Conversely, knockdown of Txnip (which would be expected to reduce protein S-nitrosylation) (Fig. 6C right panel and 6E) reduced insulin uptake (Fig. 6C left panel and 6D). These data indicate that the NO-mediated protein S-nitrosylation plays a critical role in regulation of insulin uptake.

The mechanism by which protein S-nitrosylation regulates insulin uptake is not clear. Protein-tyrosine phosphatase 1B (PTP1B) plays a critical role in inhibition of intracellular insulin signaling. Insulin signaling begins with tyrosine phosphorylation of the insulin receptor and subsequent tyrosine phosphorylation of its primary substrates, the IRS proteins. PTP1B dephosphorylates these proteins to reduce their activity. In this way intracellular insulin signaling is balanced. Increasing evidence has demonstrated that
PTP1B activity under normal metabolic condition is tightly regulated by oxidation/reduction reactions including S-nitrosylation/denitrosylation involving the cysteine thiol moiety required for catalysis (for review see (41)). Moreover, our and other’s previous studies (12;14;26) have demonstrated that inhibiting insulin signaling significantly reduces insulin transport, whereas enhancing insulin signaling by inhibition of PTP1B significantly increases insulin uptake (12). In the present study, we used an in situ detection of protein S-nitrosylation combined with the immunohistochemical staining for PTP1B in ECs freshly harvested from rat aorta and found that NO not only increased protein S-nitrosylation but also increased the co-localization of S-NO and PTP1B as well as enhancing insulin-stimulated Akt phosphorylation at Ser\textsuperscript{473}, a marker for enhanced insulin signaling via the IRS1/2-PI3K-Akt pathway. We also noted that co-treatment with SNP and insulin inhibited PTP1B activity in cultured aortic endothelial cells. These new findings, combined with our previous report (12) that the inhibition of PTP1B enhanced insulin uptake, indicate that the nitric oxide’s effects observed in present study may be, at least in part, mediated by the inactivation of PTP1B possibly via its S-nitrosylation (Fig. 8). Nitric oxide’s ability to completely restore insulin uptake that had been inhibited by Src kinase inhibitor PP1 is consistent with this hypothesis as Src activity is tightly regulated by PTP1B (41). Further studies are warranted to thoroughly clarify this complex mechanism. Taken together our data suggest that several pathways of NO-regulated signaling are required for NO-stimulated insulin uptake.

In summary, we report what we believe to be the first observations that NO can directly act on arterial endothelial cells to promote insulin uptake and trans-endothelial transport
under both physiological and pathophysiological conditions. Beyond that, our findings suggest a significant role for protein S-nitrosylation in NO’s action on insulin uptake and transport. Inhibition of PTP1B possibly induced by its S-nitrosylation appears to significantly contribute to this.

ACKNOWLEDGMENTS

This work was supported by research grants from the NIH (DK057878 and DK073059) and ADA 11-BS6 to EJB.

No potential conflicts of interest relevant to this article were reported.

H.W. designed the study, conducted experiments, performed data analyses and wrote the manuscript. A.W. and K.A. conducted experiments. E.B. contributed to discussion and reviewed/edited the manuscript. H.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References


2. Vincent, MA, Clerk, LH, Lindner, JR, Klibanov, AL, Clark, MG, Rattigan, S, Barrett, EJ: Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake in vivo. Diabetes 53:1418-1423, 2004


Figure legends

Figure 1

Nitric oxide directly promotes EC FITC-insulin uptake. bAECs were serum-starved for 6 h, then pretreated with or without 0.5 mmol/L L-arginine or 0.5 mmol/L D-arginine ± 100 μmol/L L-NAME for 30 min followed by 50 nmol/L FITC-insulin ± 0.3 μmol/L SNP or vehicle for 30 min before fixation and immunocytochemical staining. A, Effects of L-NAME on FITC-insulin uptake. * p<0.05 compared to EBM+FITC-insulin but p>0.05 compared to EBM (incubated in the basal medium without FITC-Inosulin). B, representative confocal images of bAECs stained for FITC from 3 independent experiments. C, the histograms indicate the dose response of FITC-insulin uptake to SNP treatment. * and ** p<0.001 compared to all remaining groups. D, quantification of the fluorescent intensity of FITC for each experimental condition indicated in the confocal
images. * p<0.05 compared to EBM, p<0.01 compared to SNP group and p<0.001 compared to L-ARG (L-arginine), but p>0.05 compared to D-ARG and L-ARG+LNA(L-NAME) groups. **p<0.001 compared to all remaining groups. E, Effects of L-NAME on SNP-stimulated increase of FITC-insulin uptake. * p<0.01 compared to remaining groups.

Figure 2
SNP promotes insulin trans-endothelial transport. 200 pmol/L ^{125}\text{I}-insulin alone or in the presence of either 0.3 µmol/L SNP or vehicle was added into the top chamber of transwell plates and samples were removed from the bottom chamber of the transwell plates at both 10min and 60 min for measurement of ^{125}\text{I}-insulin transported. Percent transport of total added ^{125}\text{I}-insulin at 60 min was calculated. * p<0.05 compared to both EBM group and vehicle control (n=3).

Figure 3
Effects of SNP on FITC-insulin uptake by ECs pre-treated with inhibitors of insulin action. bAECs were serum-starved for 6 h, then pretreated with either 100 nmol/L wortmannin (WOT, panel B or WORT, panel A) or 10 µmol/L PP1 (PP1, panel C) for 30 min followed by 50 nmol/L FITC-insulin ± 0.3 µmol/L SNP or vehicle (VEC) for 30 min before fixation and immunocytochemical staining. A, representative confocal images of bAECs stained for FITC from 3 independent experiments. B and C, quantitative analysis of cellular insulin uptake for each experimental conditions. #,*,** p<0.05 compared to remaining groups (B); *,# p<0.05 compared to remain groups (C). D, representative
confocal images of bAECs that were serum-starved ± 5 ng/ml TNFα for 6 h followed by 50 nmol/L FITC-insulin ± 0.3 µmol/L SNP or vehicle for 30 min before fixation and immunocytochemical staining from 3 independent experiments. E, quantitative analysis of the cellular insulin uptake presented in the panel D. *,# p<0.05 compared to remaining groups and compared each other.

**Figure 4**

**Effects of cGMP analogue and ODQ on insulin uptake.** bAECs were serum-starved for 6 h, then pretreated with either 8-Br-cGMP (0.01, 0.1, 2 or 4 mmol/L) for 5 min or ODQ (2 µmol/L or 20 µmol/L or vehicle) for 15 min followed by FITC-insulin 50 nmol/L ± 0.3 µmol/L SNP for 30 min before fixation and immunocytochemical staining. A, representative confocal images of bAECs stained for FITC from 3 independent experiments. B, the histograms indicate the dose response of FITC-insulin uptake to Br-cGMP treatment. * p<0.01 compared to the remaining groups. C, quantitative analysis of cellular insulin uptake for each experimental condition. * and ** p<0.05 compared to remaining groups.

**Figure 5**

**Effects of Insulin and/or SNP on EC general protein S-nitrosylation and specific PTP1B S-nitrosylation and activity.** Freshly harvested rat aortic endothelial were used for in situ detection of protein S-nitrosylation (S-NO) (panel A upper row, green, revealed by fluorescein) combined with immnocytochemical staining for PTP1B (Red, revealed by Cy3). A, representative confocal images from single optical sections. Arrows
point out the co-localization of S-NO and PTP1B. B, the histograms that quantify S-NO. * and **p<0.001 compared to the remaining groups. C, gives PTP1B activity of the bAECs that were serum-starved for 6 h followed by incubation with 0.3 µmol/L SNP or vehicle with 50 nmol/L insulin for 30 min before being immunoprecipitated with anti-PTP1B antibody and the tyrosine phosphatase activity measured in the immunoprecipitate, *p<0.05 compared to either vehicle or control group. Results were sum of 3 independent experiments with triplicates for each experiment.

Figure 6

Effects of knockdown of thioredoxin-interacting protein (Txnip) on insulin uptake.
bAECs were transfected with either Txnip siRNA or scrambled control siRNA. Forty-eight h after the transfection, cells were processed for western blotting or cells were serum-starved for 6 h followed by incubation with or without 50 nmol/L FITC-insulin (FITC-Ins) ± 0.3 µmol/L SNP for 30 min before they were fixed and doubly stained using anti-FITC (red, revealed by Cy3, left panel) and anti-Txnip (green, revealed by Cy2, right panel) primary antibodies. A, representative Western blots. Caveolin-1 was used as a control to assess non-specific off-target effects of siRNA silencing. GAPDH was used as a loading control. B, mean values for the ratio of Txnip to GAPDH measured by western blotting. * p<0.01 compared to scrambled control. C, representative confocal images from single optical sections. D and E, the histograms indicate the quantitation of FITC-insulin (D) and Txnip (E) fluorescence intensity observed in 3 experiments. * and ** p<0.05 compared to remaining groups; # p>0.05 compared EBM+FITC-insulin group (FITC-insulin treated without transfection of siRNA). F-H, Fresh rat aortic endothelial
cells were transferred to polylysine-coated coverslips (see Methods section for details). After 1h stabilization, these cells were treated with ether 0.3 µmol/L or 30 µmol/L SNP or vehicle for 30 min before fixation and immunocytochemical staining for both Txnip (Green, revealed by Cy 2) and FITC (Red, revealed by Cy3). F, representative confocal images of single optical sections from 3 independent experiments. G and H, the histograms indicate the quantitation of Txnip (G) and FITC-insulin (H). *and #p<0.05 compared to remaining groups. I, bAECs were serum-starved for 6 h followed by incubation with 0.3 µmol/L SNP or 50 nmol/L insulin or 50 nmol/L insulin with or without SNP for 30 min before processed for western blotting of Txnip. The representative blots from 3 independent experiments were shown. GAPDH was used as a loading control. J, bAECs were serum-starved for 6 h followed by incubation with 50 nmol/L insulin with or without 0.3 µmol/L SNP for 30 min. Cells were then processed for real time RT-PCR (n=3; no statistical difference between treatments).

Figure 7

Effects of SNP on FITC-insulin uptake and Akt phosphorylation at Ser\(^{473}\) in both chow and HFD-fed rats Rat aortic endothelial cells were transferred to coverslips, and then treated with 50 nmol/L FITC-insulin with either 0.3 µmol/L SNP or vehicle for 30min followed by immunocytochemical staining for pAkt (Green, revealed by Cy2) and FITC (Red, revealed by Cy3). A, representative confocal images of single optical sections. B and C, the histograms indicate the quantitation of FITC-insulin; *p>0.05 compared to HFD control but p<0.05 compared to remaining groups; # and **p<0.05 compared to remaining groups (B) and pAkt; * and # p<0.05 compared to CHOW FITC-
insulin, CHOW FITC-insulin+SNP and HFD FITC-insulin+SNP groups; **p<0.05 compared to remaining groups (C); n=4 for each group.

**Figure 8**

**Summary of the new mechanistic findings of present study.** Nitric oxide stimulates S-nitrosylation of PTP1B which decreases its enzymatic activity thereby limiting the de-phosphorylation of tyrosine residues on key insulin signaling proteins (IR, IRS1/2 and Src), which facilitates insulin own trans-endothelial transport (42). Txnip promotes NO-mediated S-nitrosylation of PTP1B through inhibition of denitrosylases leading to the inhibition of PTP1B and activation of insulin signaling.
Figure 1

A

FITC-insulin fluorescence intensity

EBM  EBM  L-NAME

B

FITC-insulin fluorescence intensity

EBM  EBM  L-NAME

C

FITC-insulin fluorescence intensity

EBM  EBM  L-NAME

D

FITC-insulin fluorescence intensity

EBM  EBM  L-NAME

E

FITC-insulin fluorescence intensity

EBM  EBM  L-NAME
Figure 2

The figure shows the percentage transport of 125I-insulin under different conditions at 10 min and 60 min. The bars represent the transport values for EBM, SNP, and vehicle groups. * indicates statistically significant differences.
Figure 3

A

EBM

FITC

WORT

WORT+SNP

WORT+VEC

B

FITC-insulin fluorescence intensity

EBM EBM WOT WOT WOT +SNP +VEC

FITC-insulin

C

FITC-insulin fluorescence intensity

EBM EBM PP1 PP1 PP1 +SNP +VEC

FITC-insulin
Figure 3 cont

D

EBM
FITC
TNF
TNF+SNP
TNF+VEC

50μm

E

FITC-insulin fluorescence intensity

EBM EBM TNF TNF +SNP TNF +VEC

FITC-insulin

* #
Figure 4

A

EBM

INS

INS+cGMP10

INS+cGMP100

INS+SNP

INS+SNP+ODQ2

INS+SNP+ODQ20

INS+SNP+VEC

B

FITC-insulin fluorescence intensity

EBM

cGMP 0.01
cGMP 0.1
cGMP 2
cGMP 4

SNP

SNP+ODQ2

SNP+vehicle

FITC-insulin fluorescence intensity

EBM

cGMP 0.01
cGMP 0.1
cGMP 2
cGMP 4

SNP

SNP+ODQ2

SNP+vehicle
Figure 5 cont

### Figure 5B

**S-NO fluorescence intensity**

- **Control**
- **0.3 M SNP**
- **Insulin**
- **0.3 M SNP + insulin**

- **NS**
- *****

### Figure 5C

**PTP1B activity (pmol PO4)**

- **Control**
- **Vehicle**
- **0.3 M SNP + insulin**
Figure 6

A

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B

Ratio of Txnip to GAPDH

![Graph showing ratio of Txnip to GAPDH for Txnip siRNA and Control siRNA]
Figure 6 cont

C

FITC

Txnip

EBM

INS

INS+SNP

INS+SNP+TXsiRNA

INS+SNP+CTsiRNA

D

FITC-insulin fluorescence intensity

0.0

0.5

1.0

1.5

2.0

2.5

EBM

EBM

SNP

SNP+TXsiRNA

SNP+CTsiRNA

FITC-insulin

*

**

#

**

NS

E

Txnip fluorescence intensity

0.0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

EBM

EBM

SNP

SNP+TXsiRNA

SNP+CTsiRNA

FITC-insulin

* 

# 

** 

** 

NS
Figure 7

A

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B

- **CHOW**
  - Control
  - FITC-insulin
  - FITC-insulin + 0.3\(\mu\)M SNP
- **HFD**
  - Control
  - FITC-insulin
  - FITC-insulin + 0.3\(\mu\)M SNP

C

- **CHOW**
  - Control
  - FITC-insulin
  - FITC-insulin + 0.3\(\mu\)M SNP
- **HFD**
  - Control
  - FITC-insulin
  - FITC-insulin + 0.3\(\mu\)M SNP

* \(p < 0.05\)
** \(p < 0.01\)
# \(p < 0.001\)
**SUPPLEMENTAL FIGURES AND FIGURE LEGENDS**

**Supplemental figure 1**

**A**

**B**

**Effects of SNP on genistein or PD98059-induced inhibition of FITC-insulin uptake.** bAECs were serum-starved for 6 h, then pretreated with either 50 μM PD 98059 (PD) or 50 μmol/L genistein (GENI) for 30 min followed by 50 nmol/L FITC-insulin ± 0.3 μmol/L SNP or vehicle for 30 min before fixation and immunocytochemical staining. **A and B**, quantitative analysis of cellular insulin uptake for genistein (A) or PD 98059 treatment (n=5). * p<0.05 compared to EBM or GENI+FITC-insulin or GENI+ vec (vehicle control) group but p>0.05 compared to GENI+SNP+FITC-insulin group (A); #, *, ** p<0.05 compared to remain groups (B).
Intracellular cGMP level assay. bAECs were serum-starved for 6 h, pretreated with or without 200 µmol/L isobutylmethylxanthine (IBMX) for 10 min, then treated with insulin plus either 0.3 µmol/L or 3 µM or 30 µmol/L SNP or vehicle or EBM (basal culture medium) for 30 min before processed for cGMP level and protein level assay. No significant differences were found between groups (p>0.05; one way ANOVA).
Metabolic characteristics of the rats on either HFD or regular chow diet. A, the body weights; *p<0.05. B, serum glucose concentrations. C, serum insulin concentrations; *p<0.001. D, serum triglycerides (TG); #p=0.057. n=4 for each group.