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Endogenous NO Upon Estradiol-17β Stimulation and NO Donor Differentially Regulate Mitochondrial *S*-Nitrosylation in Endothelial Cells

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Adduction of a nitric oxide (NO) moiety (NO$^\bullet$) to cysteines termed as S-nitrosylation (SNO) has emerged as a crucial mechanism for NO signaling crucial for mediating the vascular effects of estrogens. Mitochondrion is a known vascular risk factor; however, the effects of estrogens on mitochondrial SNO are incompletely understood. In this study we determined the effects of estradiol-17β (E2β) on mitochondrial protein SNO in primary human umbilical vein endothelial cells and compared the mitochondrial *nitroso*-proteomes in E2β- and a NO donor *S*-nitrosoglutathione (GSNO)-treated cells using a proteomics approach. Treatment with 10 nM E2β and 1 mM GSNO for 30 minutes significantly increased the levels of mitochondrial SNO-proteins. Subcellular localization of SNO-proteins showed mitochondria as the major cellular organelle for protein SNO in response to E2β and GSNO. E2β stimulated mitochondrial endothelial nitric oxide synthase (eNOS) phosphorylation and mitochondrial protein SNO that was enhanced by overexpression of mitochondrion or Golgi, but not membrane targeting eNOS constructs. We identified 11, 32, and 54 SNO-proteins in the mitochondria from the untreated, E2β-, and GSNO-treated human umbilical vein endothelial cells, respectively. Comparisons of the *nitroso*-proteomes revealed that common and different mitochondrial SNO-proteins were affected by endogenous NO on E2β stimulation and exogenous NO from donor. These SNO-proteins were associated with various mitochondrial functions, including energy and redox regulation, transport, iron homeostasis, translation, mitochondrial morphology, and apoptosis, etc. Collectively, we conclude that estrogens rapidly stimulate protein SNO in endothelial mitochondria via mitochondrial eNOS, providing a mechanism for mediating the vascular effects of estrogens. (*Endocrinology* 155: 3005–3016, 2014)

Endothelial cells, lining the inner surface of all blood vessels throughout the body, play a key role in vascular health largely by stimulating nitric oxide (NO) production via endothelial NO synthase (eNOS) because NO possesses potent antiinflammatory, antiapoptotic, antithrombotic, and antioxidant effects (1–3). Formation of cyclic guanosine monophosphate (cGMP) well known to mediate many biological functions of NO, including vascular remodeling, vessel relaxation, platelet aggregation, etc. (4); however, many biological activities of NO are

cGMP independent (5). Once synthesized, NO can be rapidly converted to other reactive nitrogen species (RNS) such as nitrogen trioxide (N_2O_3), peroxinitrite ($ONOO^-$), and nitrosoglutathione (GSNO) (6). These RNS can donate a NO moiety (NO$^\bullet$) to cysteines in peptides or proteins, a process called *S*-nitrosylation (SNO) that results in the formation of nitrothiols. This rapid, reversible, and redox-sensitive posttranslational modification has emerged as a crucial cGMP-independent signaling pathway for NO (7), which modulates the

functions of a plethora of proteins and participates in the regulation of nearly all major biological pathways (8).

NO signaling plays a key role in mediating the so-called "protective" effects of estrogens in the vasculature. Estrogens stimulate endothelial NO production via rapid eNOS activation and increased eNOS expression via specific estrogen receptor (eg, ERα and ERβ)-dependent mechanisms (9–12). Of note, the current understanding of estrogen action on endothelial cells is predominantly formulated from studies of NO biosynthesis via eNOS; however, the targets and functional sequelae of enhanced NO production by estrogen stimulation remain largely unknown. We have recently shown that estradiol-17β (E2β) rapidly stimulates protein SNO via specific ER- and eNOS-dependent mechanisms in primary endothelial cells (13, 14). These findings suggest that SNO serves as a novel mechanism in mediating the vascular effects of estrogens. By using biotin switch-based proteomics approach, we have obtained a partial estrogen-responsive endothelial *nitroso*-proteome in which many SNO-proteins are found to be mitochondrial proteins (13, 14). A few studies have also recently shown that SNO is important for the cardioprotective effects of estrogens in ischemic heart because ischemic preconditioning results in increased SNO of a variety of proteins that are mostly mitochondrial proteins (15, 16). These findings suggest that mitochondrion may be the major organelle that is affected by estrogens via SNO.

Mitochondrion is the organelle for synthesizing ATP and energy production; with reactive oxygen species (ROS) as major byproducts it also plays a crucial role in various key cellular functions such as oxidative phosphorylation, oxidative stress, and apoptosis (17). Mitochondrion is the primary organelle that NO targets because NO exposure results in substantial changes in mitochondrial respiration (18). Numerous studies have previously shown that estrogens have profound effects on mitochondrial functions in endothelial cells, including mitochondria biogenesis (19), energy generation (20), aging (21, 22), etc. It has been demonstrated that NO derived from mitochondrial eNOS plays a major role in mediating the mitochondrial effects of estrogens in endothelial cells (20, 23, 24); however, the mechanisms post-NO biosynthesis such as SNO, by which estrogens regulate mitochondrial functions, are incompletely understood. Although a few mitochondrial SNO-proteins have been identified (13, 14, 25–27), a large-scale analysis of estrogen-responsive endothelial mitochondrial *nitroso*-proteome has not been performed to date. Therefore, this study was performed to develop a comprehensive proteomics approach for analyzing global changes in the estrogen-responsive mitochondrial SNO-proteins in endothelial cells.

Materials and Methods

Chemicals and antibodies

Estradiol-17β (E2β), sodium ascorbate, HEPES, diethylene triamine pentaacetic acid, neocuproine, copper chloride, fatty acid-free BSA, methanol, N-ethylmeleimide methyl methanethiosulfonate, 3-(3-Cholamidopropyl)dimethylammonio-1-propanesulfonate, sodium dodecyl sulfate (SDS), dimethyl sulfoxide, and all other chemicals unless specified, were from Sigma. N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP) was from Thermo Scientific. S-Nitrosoglutathione (GSNO) was from BioVision. Trypsin Gold (mass spectrometry grade) was from Promega Corp. Antibiotin antibody was from Cell Signaling Technology. 2-(6-biotinoyl-amino-hexanoyl amino) ethylmethanethiosulfonate-Texas red was from Toronto Research Chemicals. Anti-β-actin monoclonal antibody was from Ambion. Antibodies against eNOS and glyceraldehyde 3-phosphate dehydrogenase and ser[1177] phosphorylated eNOS were from Santa Cruz Biotechnology, Inc. Antibodies against lamin B and cytochrome *c* oxidase IV were from Abcam. Oregon-Green wheat germ agglutinin, alexa[488]-Con A, MitoTracker-Green FM, prolong Gold antifade reagent, MCDB131, and M199 were from Invitrogen.

Cell culture and treatment

Human umbilical cord vein endothelial cells (HUVECs) were isolated by collagenase digestion as described previously (13). Umbilical cords were collected from the University of California Irvine Medical Center Hospital (Orange, CA) and approved by the Institutional Review Boards. Cells were cultured in endothelial cell medium (purchased from ScienCell) containing 5% fetal bovine serum (ScienCell) and supplemented with 1% antibiotics and 1% endothelial cell growth supplement and used within 5 passages. Cells at approximately 70% confluence were cultured in serum-free M-199 (phenol red-free M-199 containing 0.1% fatty acid-free BSA, 1% fetal calf serum, and 25 mM HEPES) for 16 hours. After 1-hour equilibration with fresh M199–0.1% BSA-25 mM HEPES, the cells were treated with E2β for 20–30 minutes. Ethanol (final concentration <0.01%) was used to dissolve E2β, which did not alter cellular responses. GSNO was used as a NO donor for a positive control.

Biotin-switch (BST), SDS-PAGE, and immunoblotting

Biotin switch was performed as previously described (13). Briefly, HUVECs (~4 × 10^6 cells) or the purified mitochondrial proteins were lysed in blocking buffer (25 mM HEPES, pH 7.7; 1 mM diethylene triamine pentaacetic acid; 0.1 mM neocuproine; 50 mM N-ethylmeleimide; and 2.5% SDS). The protein content of the samples was determined and was adjusted to 0.6 mg/mL. The samples (50 μg/group) were transferred to 1.5-mL amber Eppendorf tubes and were incubated at 50°C for 30 minutes in the dark, followed by precipitation with cooled (−20°C) acetone (1:3, vol/vol) and incubation at −20°C for 2 hours. Following centrifugation (12 000 × *g*, 10 minutes), the acetone-precipitated proteins were washed once with 75% cold acetone, and then resuspended in labeling buffer (25 mM HEPES, pH 7.7; 30 mM sodium ascorbate; 0.1 μM CuCl$_2$; 0.4 mM N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide; and 1% SDS). After adjusting the protein content to 0.6 mg/mL, the samples were incubated at 37°C for 1 hour in the dark with occa-

sional agitation. Finally, the samples were mixed with SDS sample buffer (without dithiothreitol or 2-mercaptoethanol) for SDS-PAGE and immunoblotting analyses. Total SNO-proteins of each sample were detected by immunoblotting with antibiotin antibody. All proteins in a lane were summed as the level of SNO-proteins of the sample.

Subcellular localization of SNO-proteins by fluorescence microscopy

SNO-proteins were detected in intact cells by a modified BST protocol as previously described (13, 28). Briefly, HUVECs were cultured on glass coverslips and then cultured in serum-free M-199 when reaching 70% confluence and treated with E2β or GSNO for 20–30 minutes. The cells were washed with cold PBS and fixed with methanol at −20°C for 15 minutes. Free thiols were blocked with 0.2 M methyl methanethiosulfonate in HEN/methanol buffer (80% methanol; 100 mM HEPES, pH 7.7; 1 mM EDTA; and 0.1 mM neocuproine) at 50°C for 30 minutes in darkness. After 3 washes with 100 mM HEPES/80% methanol, the cells were incubated with 0.2 M ascorbate and 0.2 μM 2-(6-biotinoyl-amino-hexanoyl amino) ethylmethanethiosulfonate-Texas Red in 100 mM HEPES/80% methanol at 37°C for 1 hour in the dark. After extensive washing with methanol, the cells were washed with PBS and then incubated with 1% BSA in PBS for 10 minutes. After a brief washing with PBS, the cells were incubated with Oregon-Green-wheat germ agglutinin, Alexa[488]-concanavalin A, or MitoTracker-Green FM for 50 minutes at room temperature to label Golgi apparatus, endoplasmic reticulum, and mitochondria, respectively. After washing with PBS, the cells were mounted with Prolong Gold antifade reagent and examined under a fluorescence microscope; fluorescence images were captured by a Hamamatsu charge-coupled device camera using SimplePCI software (Compix).

Mitochondrion extraction

Mitochondrion extraction was performed by using the Mitochondrion Isolation Kit (Miltenyi Biotec) with the manufacturer's instructions. Briefly, HUVECs (~1 × 10^{7} cells) were quickly washed and trypsinized at 37°C for 3 minutes. The cells were resuspended in 1 mL ice-cold Lysis Buffer and were homogenized in a Dounce homogenizer (80 strokes). The lysates were transferred into a 15-mL tube with 9 mL of ice-cold Separation Buffer. After incubation with 50 μL microbeads for 1 hour at 4°C, which were precoated with monoclonal antibody against the translocase of outer mitochondrial membrane 22, the microbead-bound mitochondria were purified on the LS column using the magnetic MidiMACS Separation Unit. The purified mitochondria were pelleted by centrifugation (13 000 × g, 2 minutes) and resuspended in 100 μL of Storage Buffer and stored at −80°C for further analysis. Protein content was determined by the Piece BCA protein assay kit.

Mitochondrion and plasma membrane eNOS targeting and cell transfection

The pCDNA3 plasmids carrying the wild-type (WT), plasma membrane targeting, and mitochondrion targeting eNOS cDNAs were kindly provided by Dr. Stephen Felton (Medical College of Georgia). The plasma membrane targeting eNOS (CAAX-eNOS) was reconstructed by fusion of the 15-amino acid membrane targeting sequence from K-ras (CAAX) in-frame

to the C terminus of the cytosolic myristoylation site mutant eNOS (G2A-eNOS) that is catalytically identical to WT-eNOS as previously described (29). The mitochondrion-targeting eNOS (Mito-eNOS) was constructed by fusion of a targeting sequence derived from human cytochrome-c oxidase subunit VIII to G2A-eNOS as previously described (30). HUVECs were seeded at a density of 1.5 × 10^{5} cells per well in a 6-well plate and transfected on the next day with the plasmids carrying cDNAs for WT-, Mito-, and CAAX-eNOS using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

To assess the activity of the overexpressed eNOS on S-nitrosylation, the ratio of SNO-proteins in organelle-eNOS-transfected and total SNO-proteins was calculated by the following equation:

$$\text{Ratio} = \frac{\text{Total SNO} - \text{Basal SNO}}{\text{Total eNOS} - \text{Basal eNOS}} \Big/ \frac{\text{Basal SNO}}{\text{Basal eNOS}}.$$

Basal eNOS is endogenous eNOS in nontransfected cells; basal SNO is constitutively SNO-proteins; [Total eNOS − Basal eNOS] stands for the overexpressed organelle-specific eNOS; [Total SNO − Basal SNO] stands for SNO mediated by overexpressed eNOS; $\left[\dfrac{\text{Basal SNO}}{\text{Basal eNOS}}\right]$ was used to normalize the data.

Trypsin digestion, avidin capture of SNO-peptides, and liquid chromatography (LC) mass spectrometry (MS)[n] analysis

Protein digestion and avidin capture were performed as previously described (31). Briefly, the biotin-labeled protein samples (1 mg) by BST were precipitated by acetone and resuspended in 200 μL of 50 mM ammonium bicarbonate/1 M urea. The samples were incubated with trypsin (20 μg) at 37°C for 18 hours, followed by incubation with NeutrAvidin beads (25 μL) prewashed in 50 mM ammonium bicarbonate at room temperature for 1 hour. The beads were washed 3 times with 1 M ammonium bicarbonate, followed by 3× washes with H_2O. Peptides were eluted from the beads with 100 μL of 0.4% trifluoroacetic acid/30% acetonitrile at room temperature for 30 minutes and then eluted one more time with 50 μL of the solution. The samples were dried using a SpeedVac and resuspended in 4% acetonitrile/0.1% formic acid for mass spectrometric analysis. Complete drying was avoided to diminish sample loss and thiol oxidation.

LC multistage tandem MS (MS/MS) analysis of cross-linked peptides was carried out using an LTQ-Orbitrap XL MS (Thermo Scientific) coupled with an Eksigent NanoLC system (Eksigent). Briefly, the LC analysis was performed using a capillary column (100 μm i.d. × 150 mm long) packed with C18 resins (GL Sciences) and the peptides were eluted using a linear gradient of 2%–40% B in 35 minutes; (Solvent A: 100% H_2O/0.1% formic acid; Solvent B: 100% acetonitrile /0.1% formic acid). For LC MS/MS analysis, a cycle of one full Fourier transform ion cyclotron resonance mass spectrometry scan mass spectrum (350–1800 mass to charge ratio [m/z], resolution of 60 000 at m/z 400) was followed by 10 data-dependent MS/MS acquired in the linear ion trap with normalized collision energy (setting of 35%). Target ions selected for MS/MS were dynamically excluded for 30 seconds.

The MS data was extracted and analyzed as previously described (32). Monoisotopic masses of parent ions and corresponding fragment ions, parent ion charge states, and ion intensities from LC-MS/MS spectra were extracted using in-house

software based on Raw_Extract script from Xcalibur v2.4. Following automated data extraction, the resultant peak lists for each LC-MS/MS experiment were submitted to the development version (v5.8.0) of Protein Prospector (University of California, San Francisco) for database searching using a concatenated SwissProt database composed of a SwissProt database and its randomized version (SwissProt.2010.08.10.random.concat). *Homo sapiens* was selected as the restricted species. Trypsin was set as the enzyme with a maximum of 2 missed cleavage sites. The mass tolerances for parent and fragment ions were set as 20 ppm and 0.8 Da, respectively. Chemical modifications such as protein N-terminal acetylation, methionine oxidation, N-terminal pyroglutamine, and deamidation of asparagine were selected as variable modifications. Maximal modifications on a given peptide were set as 3. The Search Compare program in Protein Prospector was used for summarization, validation, and comparison of results. To determine the expectation value cutoff that corresponds to a percent false positive (% FP) rate, the plot of the expectation values vs % FP rate for each search result was automatically obtained using the Search Compare Program. Based on these results, we chose an expectation value cutoff for all peptides corresponding to ≤1% FP.

Protein identification and canonical pathway analysis

Protein identification was based on at least 2 SNO-peptides identified by the LC- multistage tandem mass spectrometry. The biological functions of the identified proteins were analyzed using Ingenuity Pathways Analysis (version 7.1; http://www.ingenuity.com). Based on the local networks by computational algorithms, identified proteins were connected with hub proteins, forming a functional protein cross talk.

Experimental replication and statistical analysis

All experiments were repeated at least 3 times using cells from different fetuses. Data were presented as means ± SEM, and analyzed by one-way ANOVA, followed by Bonferroni test for multiple comparisons using SigmaStat 3.5 (Systat Software, Inc).
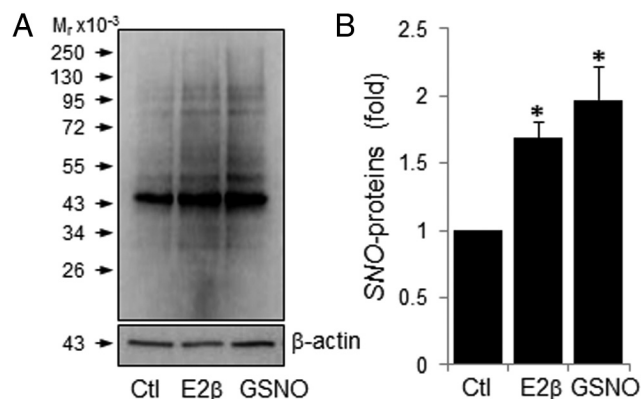
Student's t test was used for comparison of data between the 2 groups. Significance was defined as $P < .05$.

Results

E2β stimulation of protein SNO mainly occurs in the mitochondria in endothelial cells

We presented data affirming the stimulatory effects of E2β on protein SNO in endothelial cells as we recently reported (13, 14). Consistently, we detected various proteins that are constitutively nitrosylated in untreated con-

Figure 1. E2β and GSNO stimulate protein *S*-nitrosylation in HUVECs. HUVECs were treated with 10 nM E2β or 1 mM GSNO for 30 minutes. Total protein extracts were harvested and subjected to biotin-switch. The biotin-labeled SNO-proteins were analyzed by immunoblotting with antibiotin antibody. β-Actin was used as a loading control. A, Image of a representative experiment is shown; B, Plot summarized data (mean ± SEM) from 3 independent experiments. *, P < .05. Ctl, control.

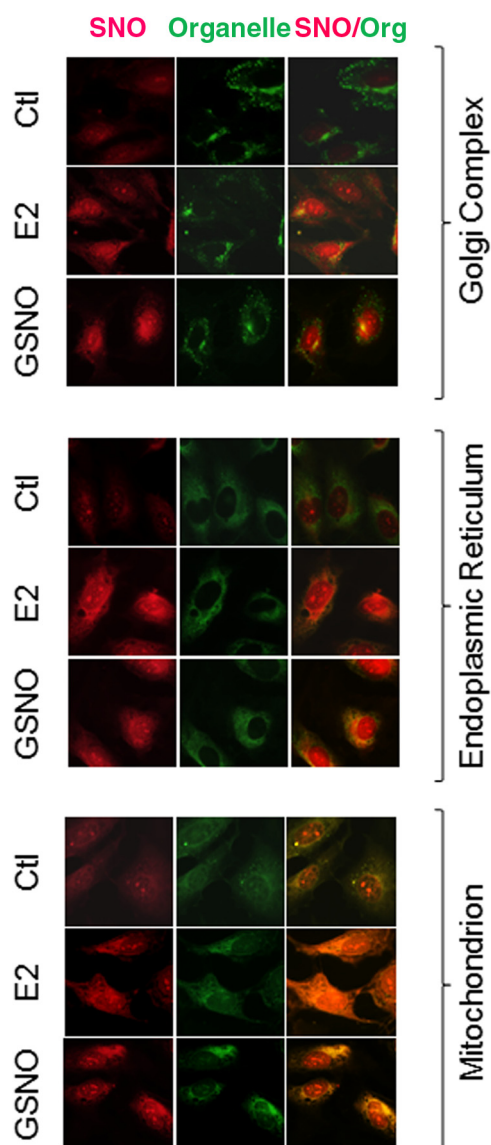**Figure 2**. In situ detection of SNO-proteins in E2β- and GSNO-treated HUVECs. HUVECs were treated with 10 nM E2β or 1 mM GSNO for 30 minutes, followed by double immune fluorescence labeling. SNO-proteins were labeled by BST; Oregon-Green-wheat germ agglutinin (WGA), Alexa[488]-concanavalin A, or MitoTracker-Green FM, were used to label Golgi apparatus, endoplasmic reticulum, and mitochondria, respectively. Ctl, control.

trol HUVECs, and treatment with E2β (10 nM) or GSNO (1 mM) for 30 minutes significantly increased the levels of SNO-proteins in HUVEC (Figure 1). To determine the subcellular location of E2β-induced SNO-proteins in endothelial cells, we detected SNO-proteins in HUVECs in situ. After treatment with or without E2β (10 nM) or GSNO (1 mM) for 30 minutes, the cells were fixed; SNO-proteins were labeled in red fluorescence by BST, and the cellular organelles, including Golgi, endoplasmic reticulum, and mitochondria were labeled in green fluorescence by using specific organelle trackers. As shown in Figure 2, in untreated control cells, constitutively nitrosylated proteins were mainly detected in the mitochondria, with weak signals in the Golgi complex and endoplasmic reticulum. Treatment with E2β or GSNO dramatically increased the red fluorescence intensities of SNO-proteins in the cytosol and nucleus of HUVECs. The E2β- and GSNO-induced

SNO-proteins in the cytosol were mainly detected in the mitochondria similar to untreated cells; however, E2β and GSNO seemed to stimulate protein SNO in the endoplasmic reticulum but not in the Golgi complex. Interestingly, increased SNO-proteins were also detected in the nucleus of E2β- and GSNO-treated cells, implicating a role of SNO in the regulation of nuclear signaling of estrogens.

E2β stimulates mitochondrial protein SNO in endothelial cells

To further demonstrate the effects of estrogens on mitochondrial protein SNO, we purified mitochondria from HUVECs by a novel method for mitochondria isolation using magnetic microbeads coated with anti-translocase of outer mitochondrial membrane 22 antibody (33). The mitochondrial preparations obtained were positive with cytochrome *c* oxidase IV, but not GAPDH, which are markers for mitochondrion and cytosol, respectively. The mitochondrial proteins purified from control, E2β- or GSNO- treated cells were subjected to BST for labeling SNO-proteins, and levels of mitochondrial SNO-proteins were determined by immunoblotting with antibiotin antibody. Constitutively nitrosylated proteins were readily detectable in the mitochondria of untreated control cells. Treatment with E2β or GSNO significantly increased total levels of mitochondrial SNO-proteins (Figure 3). In the mitochondrial preparations, immunoreactive eNOS protein was detected. Moreover, treatment with E2β stimulated eNOS phosphorylation in the mitochondria preparations in a time-dependent manner, similarly to that in whole-cell extracts (Figure 4).
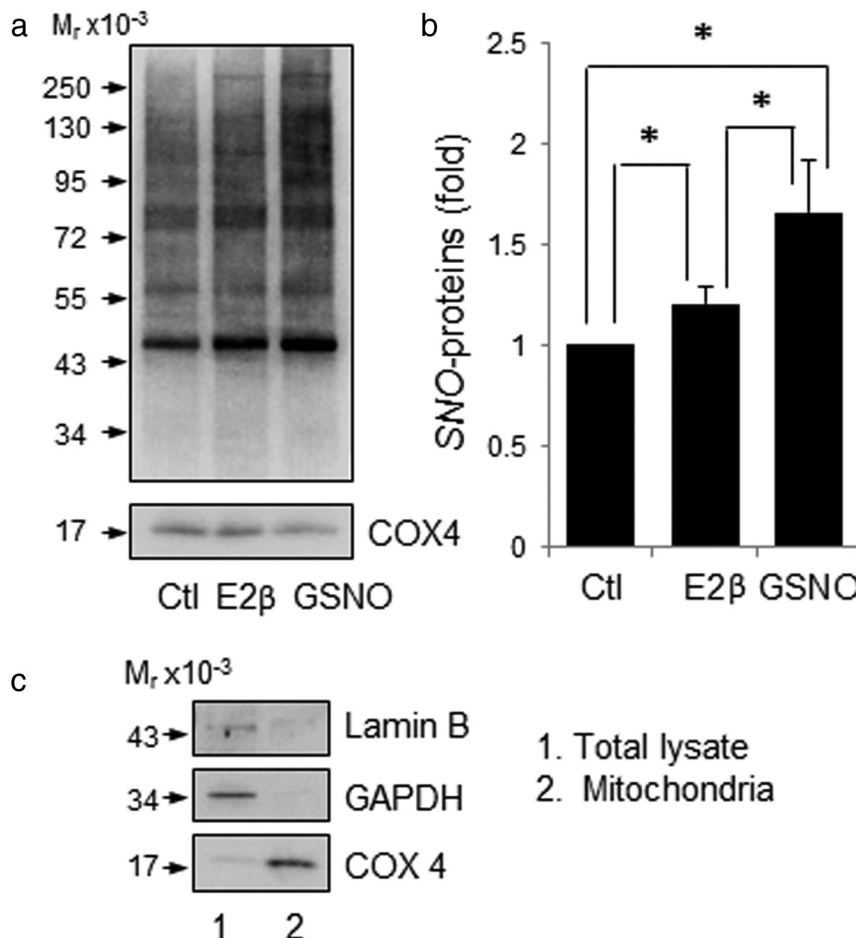
Overexpression of mitochondrion- but not membrane-targeted-eNOS enhanced E2β-stimulated protein SNO in HUVECs

When the plasmids of Mito-eNOS or CAAX-eNOS (membrane targeting) were transfected in HUVECs, overexpressed eNOS was shown to be targeted into mitochondria and plasma membrane, respectively, as previously described (29, 30). Trans-

Figure 3. E2β and GSNO stimulate mitochondrial protein *S*-nitrosylation in HUVECs. HUVECs were treated with 10 nM E2β or 1 mM GSNO for 30 minutes. a, Mitochondrial proteins were purified and subjected to biotin-switch. The biotin-labeled mitochondrial SNO-proteins were analyzed by immunoblotting with antibiotin antibody. Cytochrome *c* oxidase IV (COX IV) was used as a loading control for mitochondrion. b, Data (mean ± SEM) were summarized from 3 independent experiments. *, *P* < .05. c, Validation of mitochondria isolation from HUVECs by immunoblotting with antibodies against COX4, a mitochondrial marker. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a protein marker for cytosol.
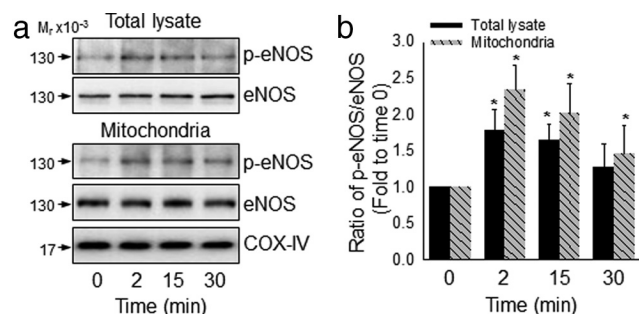
Figure 4. E2 activates mitochondrial eNOS in HUVECs. HUVECs were treated with or without 10 nM E2β for different times. Activation of eNOS in the purified mitochondria and whole-cell extracts was measured by immunoblotting with specific antibody against ser^{1177} phosphorylated eNOS. Images (a) show blots of a typical experiment and graph (b) summarized data (mean ± SEM) from 3 independent experiments. *, $P < .05$.

fections with the WT-, Mito-, and CAAX-eNOS all resulted in increased eNOS expression; however, E2β-stimulated protein SNO was enhanced by overexpression of the WT- and Mito-eNOS, but not CAAX-eNOS in HUVECs (Figure 5).

Identification of mitochondrial SNO-proteins in HUVEC by LC-MS/MS analysis

We labeled total mitochondrial SNO-proteins from untreated, E2β-, and GSNO-treated cells. Following trypsin digestion, the biotin-labeled SNO-peptides were purified for identification of mitochondrial SNO-proteins by mass spectrometry. The SNO-peptides identified were summarized in Table 1. A total of 90 SNO-peptides were identified in the purified endothelial mitochondria, including 14 from the untreated and, 41 and 83 from the E2β- and GSNO- treated HUVECs, respectively. These SNO-peptides had overlaps among the groups (Figure 6a). Eight SNO-peptides were identified in both the untreated and E2β-treated HUVECs: 10 in the untreated and GSNO-treated HUVECs and 36 in the E2β- and GSNO-treated HUVECs. Of note, 6 SNO-peptides were found in all 3 groups. Mass spectrometric analyses of these identified SNO-peptides suggest a total of 57 SNO-proteins in the mitochondrial preparations, including 11, 32, and 53 SNO-proteins in the untreated, E2β-, and GSNO-treated cells, respectively. Similarly, the SNO-proteins had overlap among the 3 groups (Figure 6b). Nine were found in both the untreated and E2β-treated HUVECs; 10 in the untreated and the GSNO-treated HUVECs; 28 in the E2β- and GSNO-treated HUVECs. Additionally, the 6 constitutive SNO-peptides with other specific peptides identified in either control, or E2, or GSNO-treated cells led to 8 possible constitutive mitochondrial SNO-proteins.

Pathway analysis

Ingenuity pathway analysis was used to explore the potential biological functions of the identified mitochon-
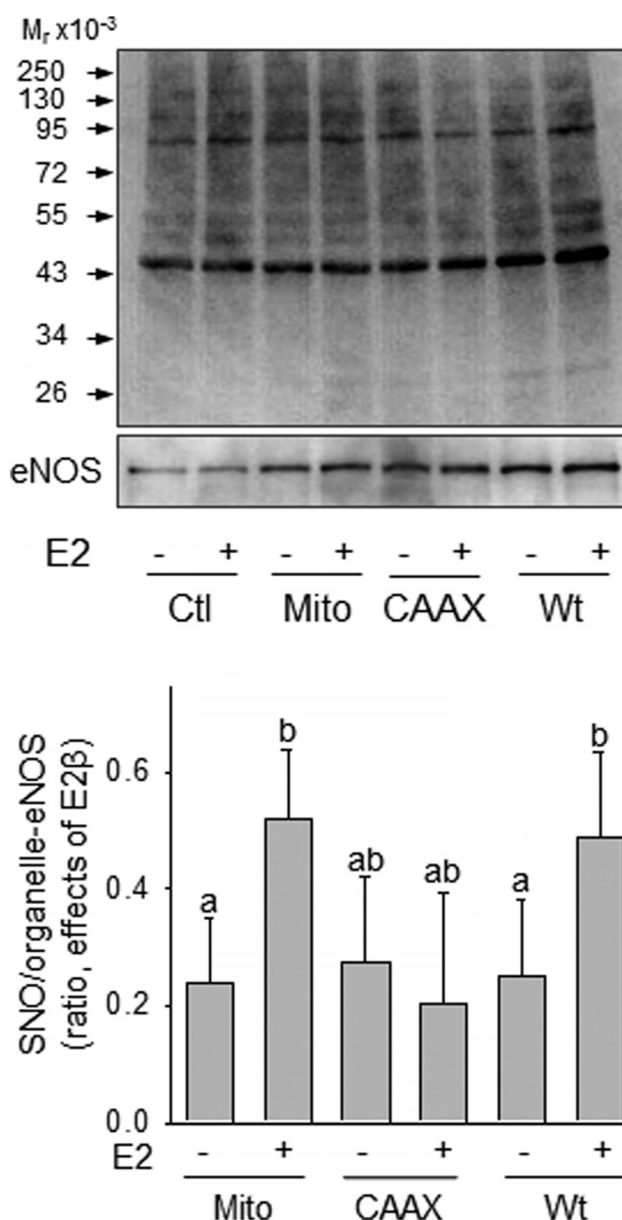
Figure 5. E2-stimulated protein SNO was enhanced by overexpression of mitochondrial or Golgi, but not membrane- targeted eNOS in HUVECs. Mitochondrial eNOS (Mito-eNOS), CAAX-eNOS, and wt-eNOS were overexpressed in HUVECs, respectively. After treatment with or without 10 nM E2β, cells were lysed and subjected to biotin-switch. The biotin-labeled SNO-proteins were analyzed by immunoblotting with antibiotin antibody. Expression levels of eNOS were determined with anti-eNOS antibody. Graph summarized data (mean ± SEM) from 3 independent experiments. Bars with different letters differ significantly ($P < .05$).Ctl, control.

drial SNO-proteins in control and E2β-treated HUVECs. Function analysis suggested that the SNO-proteins are associated with various mitochondrial functions, including nucleic acid metabolism, small molecule biochemistry, cellular assembly and organization, molecular transport, and cell morphology. Signaling pathway analysis suggested that the mitochondrial SNO-proteins identified are linked to various fundamental cellular physiologies, in-

Table 1. Mitochondrial *nitroso*-Proteome in HUVECs

Function	UniProt Identification	Protein Name	SNO-Peptide	Best Expected Value		
				Ctl	E2β	GSNO
Fatty acid oxidation	P49748	Acyl-CoA dehydrogenase	ELGAFGLQVPSELGGVGLC*NTQYAR	ND	ND	1.4E-05
	Q13011	δ (3,5)-δ (2,4)-dienoyl-CoA isomerase	YC*AQDAFFQVK	ND	ND	4.0E-04
	P40939	Trifunctional enzyme subunit α	TGIEQGSDAGYLC*ESQK	ND	6.9E-06	1.1E-07
			EVEAVIPDHC*IFASNTSALPISEIAAVSK	ND	ND	1.1E-07
			C*LAPMMSEVIR	ND	6.9E-06	1.1E-07
	Q16698	2,4-dienoyl-CoA reductase	VHAIQC*DVR	ND	ND	1.1E-03
	Q9H845	Acyl-CoA dehydrogenase family member 9	GSNTC*EVHFENTK	ND	ND	4.9E-07
	P50416	Carnitine O-palmitoyltransferase 1, liver isoform	TLETANC*MSSQTK	ND	ND	3.2E-05
			SCTTESC*DFVR	ND	3.2E-05	
	P13804	Electron transfer flavoprotein subunit α	TIYAGNALC*TVK	ND	ND	8.3E-05
			LGGEVSC*LVAGTK	ND	7.5E-04	8.3E-05
			VAQDLC*K	ND	ND	8.3E-05
	P24752	Acetyl-CoA acetyltransferase	IHMGSC*AENTAK	ND	7.2E-06	1.9E-06
	O14561	Acyl carrier protein	LMC*PQEIVDYIADK	ND	ND	1.0E-03
TCA cycle	O75390	Citrate synthase	LPC*VAAK	6.2E-06	ND	1.0E-05
	Q99798	Aconitase	VGLIGSC*TNSSYEDMGR	ND	1.8E-06	5.5E-06
	P50213	Isocitrate dehydrogenase subunit α	IEAAC*FATIK	ND	ND	2.7E-03
	P48735	Isocitrate dehydrogenase2 (NADP+), mitochondrial	C*ATITPDEAR	ND	3.5E-03	2.6E-03
	Q02218	2-oxoglutarate dehydrogenase	IC*EEAFAR	ND	1.1E-03	ND
	P40926	Malate dehydrogenase	TIIPLISQC*TPK	ND	ND	4.0E-09
			SQETEC*TYFSTPLLLGK	ND	6.8E-09	4.0E-09
			EGVVEC*SFVK	ND	ND	4.0E-09
			GC*DVVVIPAGVPR	ND	6.8E-09	4.0E-09
			GYLGPEQLPDC*LK	ND	ND	4.0E-09
	P31040	Succinate dehydrogenase flavoprotein subunit	VGSVLQEGC*GK	5.6E-06	2.8E-05	1.5E-07
			AAFGLSEAGFNTAC*VTK	ND	ND	1.5E-07
			TYFSC*TSAHTSTGDGTAMITR	5.6E-06	ND	ND
	P21912	Succinate dehydrogenase iron-sulfur subunit	CHTIMNC*TR	ND	3.7E-05	ND
	P53007	Tricarboxylate transport protein, mitochondrial	GIGDC*VR	ND	ND	5.1E-03
Electron transport chains	O95299	NADH dehydrogenase 1 α subcomplex subunit 10	VITVDGNIC*TGK	ND	1.8E-03	3.6E-03
			C*EVLQYSAR	ND	8.6E-04	3.7E-04
	O75380	NADH dehydrogenase iron-sulfur protein 6	VIAC*DGGGGALGHPK	ND	8.6E-04	3.7E-04
	P28331	NADH-ubiquinone oxidoreductase 75 kDa	VLFLLGADGGC*ITR	ND	ND	1.8E-04
	P31930	Cytochrome *b*-c1 complex subunit 1	YIYDQC*PAVAGYGPIEQLPDYNR	ND	1.7E-05	1.9E-05
			LC*TSATESEVAR	8.3E-04	1.7E-05	1.9E-05
			NALVSHLDGTTPVC*EDIGR	ND	1.7E-05	1.9E-05
	P22695	Cytochrome *b*-c1 complex subunit 2	NALANPLYC*PDYR	8.6E-05	2.6E-06	1.8E-07
			ENMAYTVEC*LR	8.6E-05	ND	1.8E-07
	P53701	Cytochrome c-type heme lyase	AYEYVEC*PIR	ND	5.9E-04	2.5E-03
	O75947	ATP synthase subunit δ	SC*AEWVSLSK	ND	3.4E-04	3.9E-04
	P56381	ATP synthase subunit ε	YSQIC*AK	ND	2.6E-03	2.9E-03
	P36542	ATP synthase subunit γ	GLC*GAIHSSIAK	ND	7.2E-05	3.7E-05
	P48047	ATP synthase subunit O	GEVPC*TVTSASPLEEATLSELK	ND	ND	5.5E-08
One carbon metabolism	Q6UB35	Monofunctional C1-tetrahydrofolate synthase	AEIDLVC*ELAK	ND	ND	6.8E-05
	P13995	Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase	IC*NAVSPDKDVDGFHVINVGR	ND	ND	6.9E-03
	P34897	Serine hydroxymethyltransferase	AALEALGSC*LNNK	ND	ND	5.0E-05
			NTC*PGDR	ND	ND	5.0E-05
			GLELIASENFC*SR	ND	3.3E-04	5.0E-05
Other metabolism	P19367	Hexokinase-1	ATDC*VGHDVVTLLR	ND	2.1E-04	1.4E-05
			TVC*GVVSR	ND	ND	1.4E-05
	P00367	Glutamate dehydrogenase 1	C*AVVDVPFGGAK	ND	ND	7.4E-05
	P43304	Glycerol-3-phosphate dehydrogenase	NYLSC*DVEVR	ND	ND	3.4E-03
	Q5JRX3	Presequence protease	C*SVNATPQQMPQTEK	ND	ND	2.2E-06
	P32322	Pyrroline-5-carboxylate reductase 1	C*MTNTPVVVR	ND	ND	2.5E-05
			VGAFTMVC*K	ND	2.5E-07	4.1E-07
	P00505	Aspartate aminotransferase	TC*GFDFTGAVEDISK	2.5E-07	4.1E-07	
			NLDKEYLPIGGLAEFC*K	ND	ND	4.1E-07
	P05091	Aldehyde dehydrogenase	LLC*GGGIAADR	ND	ND	6.8E-05
Transport	P10809	60 kDa heat shock protein	AAVEEGIVLGGGC*ALLR	2.4E-05	8.2E-07	1.1E-05
			C*EFQDAYVLLSEK	2.4E-05	8.2E-07	1.1E-05
			C*IPALDSLTPANEDQK	2.4E-05	ND	1.1E-05
	Q12931	Heat shock protein 75 kDa	NIYYLC*APNR	ND	ND	5.8E-04
	P38646	Stress-70 protein	MEEFKDQLPADEC*NK	7.5E-04	1.0E-05	6.6E-06
	Q3ZCQ8	Mitochondrial import inner membrane translocase subunit TIM50	VVVVDC*K	ND	1.6E-04	ND
	P12235	ADP/ATP translocase 1	GADIMYTGTVDC*WR	ND	ND	6.4E-09
			YFAGNLASGGAAGATSLC*FVYPLDFAR	ND	ND	
	P05141	ADP/ATP translocase 2	GLGDC*LVK160	ND	4.6E-10	6.4E-09
			KGTDIMYTGTLDC*WR257	4.8E-07	ND	6.4E-09
			YFAGNLASGGAAGATSLC*FVYPLDFAR129	4.8E-07	ND	ND
	P12236	ADP/ATP translocase 3	GLGDC*LVK	ND	4.6E-10	6.4E-09
			GADIMYTGTVDC*WR	ND	6.4E-09	
			YFAGNLASGGAAGATSLC*FVYPLDFAR	4.8E-07	ND	ND

(Continued)

Table 1.   Continued

Function	UniProt Identification	Protein Name	SNO-Peptide	Best Expected Value		
				Ctl	E2β	GSNO
	Q9Y512	Sorting and assembly machinery component 50 homolog	IC*DGVQFGAGIR	ND	ND	7.6E-04
	Q00325	Phosphate carrier protein	FAC*FER	5.0E-05	2.4E-05	ND
	Q9Y6C9	Mitochondrial carrier homolog 2	QVC*QLPGLFSYAQHIASIDGR	ND	ND	6.6E-06
	P21796	Voltage-dependent anion-selective channel protein 1	YQIDPDAC*FSAK	ND	3.2E-08	1.0E-04
			EHINLGC*DMDFDIAGPSIR	ND	ND	1.0E-04
	P45880	Voltage-dependent anion-selective channel protein 2	SC*SGVEFSTSGSSNTDTGK	ND	ND	3.2E-07
			WC*EYGLTFTEK	ND	1.9E-04	3.2E-07
			WNTDNTLGTEIAIEDQIC*QGLK	ND	1.9E-04	3.2E-07
	Q9Y277	Voltage-dependent anion-selective channel protein 3	MC*NTPTYCDLGK	ND	ND	1.0E-04
			VC*NYGLTFTQK	ND	5.5E-07	1.0E-04
			SC*SGVEFSTSGHAYTDTGK	ND	5.5E-07	1.0E-04
Transcription	Q99714	3-Hydroxyacyl-CoA dehydrogenase type-2	VDVAVNC*AGIAVASK	ND	ND	4.5E-06
			VC*NFLASQVPFPSR	ND	ND	4.5E-06
	P42704	Leucine-rich pentatricopeptide-repeat motif-containing protein, mitochondrial	LIASYC*NVGDIEGASK	ND	ND	7.2E-08
			VFNDTC*R	ND	9.9E-09	7.2E-08
			IHDVLC*K	ND	ND	7.2E-08
			C*VANNQVETLEK	ND	ND	7.2E-08
			DILIAC*R	6.5E-08	9.9E-09	ND
	P49411	Elongation factor Tu	KGDEC*ELLGHSK	ND	4.8E-06	8.2E-04
Fusion	O95140	Mitofusin-2	EQQVYC*EEMR	ND	ND	1.4E-03
Apoptosis	O60313	Dynamin-like 120-kDa protein	C*NEEHPAYLASDEITTVR	ND	ND	1.7E-04
	Q07021	Complement component 1 Q subcomponent-binding protein	ALVLDC*HYPEDEVGQEDEAESDIFSIR	ND	2.0E-04	3.9E-03

*, N-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (Biotin-HPDP)-labeled SNO-cysteines; CoA, coenzyme A; NADH, reduced nicotinamide adenine dinucleotide; ND, not detected; TCA, trichloroacetic acid.

cluding mitochondrial dysfunction, citrate cycle, oxidative phosphorylation, glyoxylate and dicarboxylate metabolism, and butanoate metabolism (Table 2). Of note, many mitochondrial SNO-proteins identified form a network for regulating cell functions (Figure 6c). According to the mitochondrial SNO-proteins identified in the resting and E2β-treated HUVECs, SNO apparently plays a critical role in the maintenance of normal mitochondrial physiology and is also important for the cell to respond to extracellular stimuli.

Discussion

In the endothelial mitochondrial *nitroso*-proteomes identified herein, we have found that 25 are E2β-responsive SNO-proteins, including 23 nitrosylated and 2 *de*-nitrosylated ones. Functional analysis classified these SNO-proteins as energy and redox metabolic enzymes responsible for carbohydrate, fatty acid oxidation, ATP synthesis, and oxidative phosphorylation. Six of them are involved in citric acid cycle, including citrate synthase, aconitase, isocitrate dehydrogenase 2, 2-oxoglutarate dehydrogenase, malate dehydrogenase, and succinate dehydrogenase iron-sulfur subunit. In addition, hexokinase-1, a glycolysis enzyme with docking sites to the outer membrane in mitochondria, is also identified as an E2β-responsive SNO-protein in the endothelial mitochondria. The identification of these mitochondrial SNO-proteins suggests that SNO is one of the mechanisms mediating the effects of estrogens in mitochondrial energy production. Notably, comparisons between the

GSNO-and E2β-responsive endothelial *nitroso*-proteomes have identified common and different *nitroso*-proteins (Figure 6). These findings show that exogenous NO from donor such as GSNO exerts different cellular responses than endogenous NO from eNOS activation upon E2 stimulation via SNO-dependent mechanisms in endothelial cells.

The respiratory chain composed of 4 electron transport chain complexes (I–IV) and the ATP synthase (complex V) are the major enzymes of mitochondria for ATP synthesis (34). NO has long been known to inhibit complex I (35), presumptively protection against mitochondrial reactive oxygen species (ROS) production and calcium overloading upon ischemic preconditioning (36). Our current study has identified the ATP synthase subunit δ, ε, and γ, as well as 2 of the complex I reduced nicotinamide adenine dinucleotide dehydrogenase 1 α subcomplex subunit 10 and reduced nicotinamide adenine dinucleotide dehydrogenase iron-sulfur protein 6, as E2β-responsive SNO-targets. Thus, the estrogen-responsive endothelial mitochondrial SNO-proteins identified herein have further shown a critical role of SNO in energy generation upon estrogen stimulation.

Estrogens increase the efficiency of energy production and decrease ROS-mediated damage of mitochondria (20). ROS, as byproducts of the process of ATP synthesis, oxidize NO to form RNS that can further induce protein SNO (37). From this standpoint, SNO may provide an important mechanism for estrogens to protect oxidative stress-mediated damage of the mitochondria.
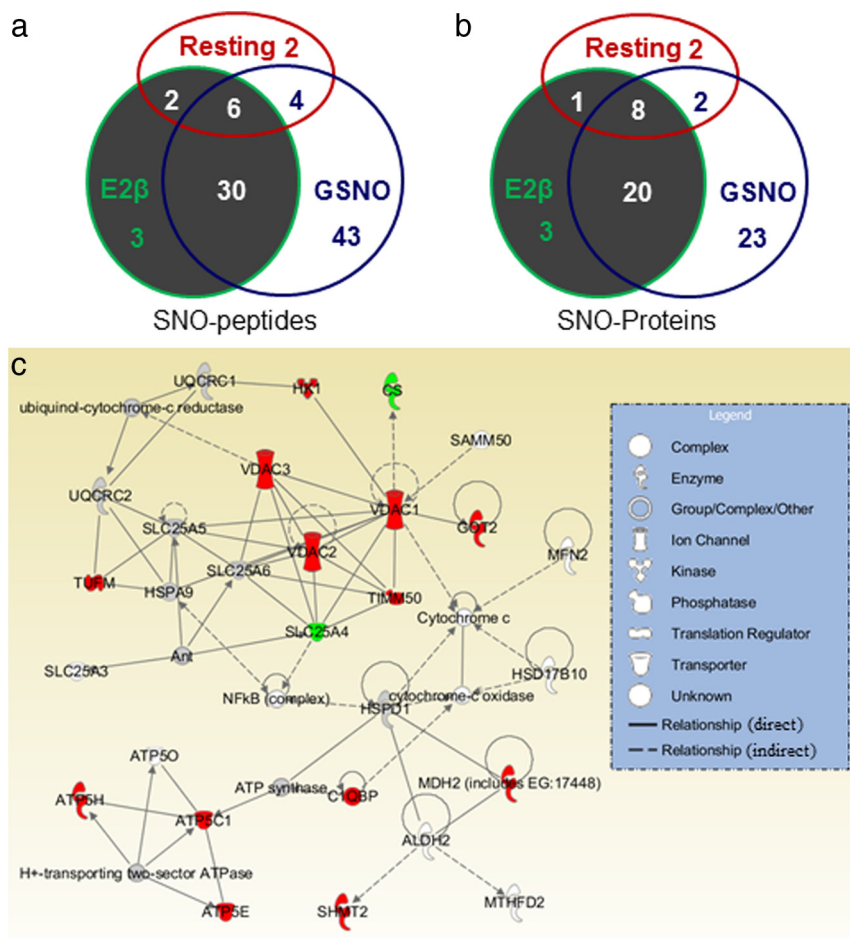
Figure 6. Mitochondrial *nitroso*-proteomes in HUVECs. Overlaps of the mitochondrial SNO-peptides (a) and SNO-proteins (b) identified from resting, and E2β- or GSNO-treated HUVECs. The mitochondrial E2β-responsive SNO-protein network (c) was generated by ingenuity pathway analysis. Proteins were represented as nodes; the biological relationship between 2 nodes is represented as a line. All lines are supported by at least one published reference. Solid lines represent a direct relationship, and dashed lines represent an indirect relationship. The red node color represents E2β stimulation. The shape of each node represents a functional class of proteins.

acid metabolism in rat heart mitochondria have been previously reported to be regulated by SNO (42).

Other E2β-responsive mitochondrial SNO-proteins identified in this study include 4 cellular transportation proteins (ie, mitochondrial import inner membrane translocase subunit TIM50 and voltage-dependent anion-selective channel protein 1, 2, and 3) and elongation factor-τ. These channels allow the diffusion of small hydrophilic molecules (43). Elongation factor-τ promotes the GTP-dependent binding of amino-acyl-tRNA to the A-site of ribosomes during protein biosynthesis (44). Therefore, identification of these SNO-targets underscores the significance of SNO in mediating mitochondrial protein synthesis and transport upon estrogen stimulation. Aspartate aminotransferase is another estrogen-response mitochondrial SNO-protein that catalyzes reversible transfer of α-amino group between aspartate and glutamate during protein synthesis (45). Physiological levels of NO are also known to promote cell survival and are antiapoptotic in endothelial cells (46). Accordingly, we have identified complement component 1 Q subcomponent-binding protein, a component of the complement system that is involved in the clearance of apoptotic cells and binds to surface blebs of apoptotic cells, followed by subsequent phagocytosis (47).

We also have shown that E2β stimulates SNO of 3 fatty acid oxidation related proteins, including trifunctional enzyme subunit α, electron transfer flavoprotein subunit α, and acetyl-CoA dehydrogenase in the mitochondria. The trifunctional enzymes catalyze the initial oxidation step and subsequent hydration of unsaturated carbons in the β-oxidation pathway (38); electron-transferring flavoprotein transfers electrons derived from the oxidation of fatty acids to ubiquinone (39); acetyl-CoA acetyltransferase plays a major role in ketone body metabolism (40). In addition, serine hydroxylmethyltransferase is also an estrogen-responsive mitochondrial SNO-protein the function of which is to support the flow of one-carbon units into the methyl cycle in embryonic mitochondria (41). Thus, these findings show that SNO targets numerous mitochondrial metabolic enzymes in endothelial cells. Consistently, 8 enzymes responsive for carbohydrate and fatty

Although the major cellular source of NO is thought to be via eNOS localized at the plasma membrane (29), previous studies also have shown that mitochondria are able to synthesize NO via eNOS at the outer membrane of mitochondria (48, 49). We also have shown that E2β stimulates eNOS ser^{1177} phosphorylation in the mitochondria in a timely dependent manner similar to whole-cell extracts. By overexpressing organelle-targeting eNOS constructs (29, 30, 50), we have observed that mitochondrion-targeting eNOS significantly enhanced E2β stimulation of protein SNO in the mitochondria, consistent with a previous report (51). In addition, we have found that mitochondria are the major organelle of SNO-proteins in E2β-treated cells. These data thus suggest that

Table 2.   Ingenuity Pathway Analysis of HUVEC Mitochondrial *nitroso*-Proteome: Signaling Pathways and Molecular Functions

Name	List of Molecules	n	Ratio	P Value
Mitochondrial dysfunction	ATP5C1, CPT1A, GPD2, HSD17B10, NDUFA10,NDUFAB1, NDUFS1, NDUFS6,OGDH, SDHA, SDHB, UQCRC1, UQCRC2	13	13/174 (0.075)	1.72E-15
Citrate cycle	ACO2, CS, IDH2, IDH3A, MDH2, OGDH, SDHA, SDHB	8	8/57 (0.14)	9.48E-14
Oxidative phosphorylation	ATP5C1, ATP5E, ATP5H, ATP5O, NDUFA10, NDUFS1, NDUFAB1, NDUFS6, SDHA, SDHB, UQCRC1, UQCRC2	12	12/159 (0.075)	1.49E-13
Glyoxylate and dicarboxylate metabolism	ACO2, CS, MDH2, MTHFD, MTHFD1 liter	5	5/112 (0.045)	1.21E-08
Butanoate metabolism	ACAT1, ALDH2, HADHA, HSD17B10, SDHA, SDHB	6	6/128 (0.047)	8.21E-08
Nucleic acid metabolism	ALDH2, ATP5C1, ATP5E, ATP5H,ATP5O, CS, GPD2, HSPD1, IDH3A, MDH2, MFN2, NDUFS, OGDH, OPA1, SLC25A5,VDAC1	16	ND	1.26E-10– 6.43E-09
Small molecule biochemistry	ALDH2, ATP5C1, ATP5E, ATP5H,ATP5O, GPD2, HSPD1, IDH3A, MDH2, MFN2, NDUFS1,OGDH, OPA1, SLC25A5, VDAC1	15	ND	1.26E-10– 6.43E-09
Cellular assembly and organization	MFN2, OPA1, SLC25A5, SLC25A6, TIMM50, VDAC1	6	ND	2.00E-07– 4.14E-06
Molecular transport	SLC25A4, SLC25A5, VDAC3	3	ND	2.00E-07
Cell morphology	GPD2, HSPD1, MFN2, NDUFAB1, NDUFS1, OPA1, SLC25A6, VDAC1	8	ND	6.38E-07

ND, undetermined.

local NO production via mitochondrial eNOS activation might be a major mechanism for E2β stimulation of mitochondrial protein SNO.

Levels of 2 SNO-proteins, ie, citrate synthase and ADP/ATP translocase 1, are decreased in E2β-treated cells, indicative of de-nitrosylation; however, levels of both are increased in GSNO-treated cells. These data suggest that endogenous NO generated by eNOS activation upon E2β stimulation and exogenous NO from donors like GSNO lead to opposite changes in some SNO-proteins, although E2β and GSNO stimulate SNO of many common mitochondrial proteins. De-nitrosylation of mitochondrial proteins upon E2β stimulation is unexpected, but not a surprise, as we have reported protein de-nitrosylation by E2β in intact endothelial cells (13, 14). However, the exact mechanisms by which E2β results in mitochondrial protein de-nitrosylation are not known. It is suspicious that some SNO-proteins can donate their NO groups to *trans*-nitrosylate other proteins (8). It is also possible that estrogens may activate one or more of the enzymes such as GSNO reductase (52, 53) and thioredoxin reductase (54), which in turn regulate dynamic protein SNO. However, these ideas need to be further investigated. There are many SNO-proteins that were stimulated only by GSNO, possibly due to the relative high concentration of GSNO used in comparison to the physiological levels of endogenous NO upon E2β stimulation (55). However, 3 E2β-stimulated mitochondrial SNO-proteins, including 2-oxoglutarate dehydrogenase, succinate dehydrogenase iron-sulfur

subunit, and mitochondrial import inner membrane translocase subunit TIM50, are not detected in the GSNO-treated cells. Regardless, comparison of the *nitroso*-proteomes between the E2β- and GSNO-treated HUVECs provides insights regarding the mitochondrial SNO targets that are affected by endogenous NO produced by E2β stimulation and exogenous NO from the NO donor GSNO.

Interestingly, the specific SNO-sites of the constitutive SNO-proteins can be altered in response to stimulation. For example, there are 3 different SNO-peptides in ADP/ATP translocase 2 identified, implicating 3 SNO-sites (Cys129/160/257) in this constitutive SNO-protein. Among them, Cys129 (YFAGNLASGGAAGATSLC*FVYPLDFAR) and Cys-257 (KGTDIMYTGTLDC*WR) were identified as the SNO-sites in the untreated HUVECs. However, these SNO-peptides were not found in the E2β-treated HUVECs, but rather a different Cys160 (GLGDC*LVK) was identified. Moreover, GSNO stimulated SNO on Cys160 and results in de-nitrosylation on Cys129. These data suggest that SNO-regulated protein function may be even more complicated because this post-translational modification may alter constitutive SNO-proteins via alterations on the SNO-sites in response to different stimuli.

There are a few limitations in our current BST-based proteomics approach for analyzing S-nitrosylation, although in theory this powerful method has the potential of extracting all SNO-proteins from a proteome with the

identification of specific SNO-sites simultaneously. However, the accuracy of protein identification is expected to be reduced because only SNO-peptides were purified and sequenced by LC-MS/MS analysis; this leads to less peptide sequence(s) obtained for protein speculation. In addition, this method can only determine the relative changes of SNO-proteins among treatments. An unbiased quantization of changes in SNO-proteins would require input of internal standards or use stable isotope labeling of amino acids in cell culture (SILAC)-based quantitative proteomics approach (56).

Altogether, the present study has confirmed our recent studies (13, 14) showing that E2β stimulates protein SNO in endothelial cells and identified mitochondrion as a major cellular organelle in which protein SNO occurs in endothelial cells upon E2β stimulation. We have further analyzed, for the first time, the E2β- and GSNO-responsive mitochondrial *nitroso*-proteomes in endothelial cells. Function analysis of the mitochondrial *nitroso*-proteomes has revealed a network of SNO-proteins that are linked to diverse mitochondrial functions. We also have shown that forced overexpression of mitochondrion-targeting eNOS increases the E2β-stimulated SNO response. In keeping with our recent studies showing that estrogen stimulation of endothelial protein SNO is mediated by eNOS-derived NO via a specific ER-dependent mechanism (13, 14), the biological functions of the mitochondrial SNO-proteins revealed by informatics analysis suggest mitochondrial SNO as an important mechanism for mediating the endothelial effects of estrogens after biosynthesis of NO. Future studies of analyzing the functional sequelae of the estrogen-responsive mitochondrial SNO-targets are warranted and are expected to pave a new avenue for the understanding of the cardiovascular effects of estrogens.

Acknowledgments

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