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Protein disulfide isomerase overexpression in vascular smooth muscle cells induces spontaneous preemptive NADPH oxidase activation and Nox1 mRNA expression: Effects of nitrosothiol exposure

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ABSTRACT

Mechanisms regulating NADPH oxidase remain open and include the redox chaperone protein disulfide isomerase (PDI). Here, we further investigated PDI effects on vascular NADPH oxidase. VSMC transfected with wild-type PDI (wt-PDI) or PDI mutated in all four redox cysteines (mut-PDI) enhanced (2.5-fold) basal cellular ROS production and membrane NADPH oxidase activity, with 3-fold increase in Nox1, but not Nox4 mRNA. However, further ROS production, NADPH oxidase activity and Nox1 mRNA increase triggered by angiotensin-II (AngII) were totally lost with PDI overexpression, suggesting preemptive Nox1 activation in such cells. PDI overexpression increased Nox4 mRNA after AngII stimulus, although without parallel ROS increase. We also show that Nox inhibition by the nitric oxide donor GSNO is independent of PDI. PDI silencing decreased specifically Nox1 mRNA and protein, confirming that PDI may regulate Nox1 at transcriptional level in VSMC. Such data further strengthen the role of PDI as novel NADPH oxidase regulator.

Enzymatic ROS generation is an inherent component of redox signaling [1–5] and a prime target for antioxidant interventions [4]. Nox family NADPH enzyme complexes are the most important source of signaling ROS in many cells, including the vascular system, in which they are involved in the pathogenesis of several diseases such as atherosclerosis, diabetes, hypertension and restenosis after injury [3,5]. Understanding mechanisms of NADPH oxidase regulation is essential in order to allow its rational therapeutic manipulation. Most studies addressed at understanding such mechanisms disclosed important issues related to structural characteristics of Nox isoforms [1–3,5], their assembling into catalytic complexes involving distinct regulatory subunits [1,2], their different locations [6] and subcellular compartmentation [7]. Such are relevant first-order regulatory steps, which integrate with second-order mechanisms related to regulatory proteins involved in cell functions closely associated to NADPH oxidase activation. Such proteins are yet poorly understood.

Recently, our laboratory identified protein disulfide isomerase (PDI)[1], a dithiol-disulfide oxidoreductase endoplasmic reticulum chaperone, as a regulatory protein associated with NADPH oxidase in vascular smooth muscle cells [8]. Down-regulation of PDI with pharmacological and molecular interventions uniformly promoted decrease in baseline NADPH oxidase activity, and particularly in its response to the VSMC NADPH agonist angiotensin-II (AngII) [8]. Moreover, PDI was identified to associate with several oxidase subunits, including p22phox, Nox1, Nox4 and Nox2 either in native VSMC or in HEK293 cells transfected with Nox isoforms [8]. Similar association and functional interaction have recently been identified in macrophages and endothelial cells [9]. Despite being located primarily in the endoplasmic reticulum lumen, where it assists redox protein folding via its isomerase activity [9,10], PDI also displays an active outward traffic through the secretory system up to cell membranes [11], where it is involved in cell surface modulation of thiol protein redox status [12], including adhesion molecules such as integrins [13]. The interaction with NADPH oxidase complex occurs at least at this level, since PDI modulators interfere with NADPH oxidase activity assays of VSMC membrane homogenates [8,9]. Thus, PDI qualifies as a prime candidate to act as regulator of a putative redox signalosome [14]. In addition, since non-phagocytic Nox activity involves significant increase in mRNA and protein expression of distinct subunits [1–3], PDI might also affect Nox regulation by modulating subunit expression. However, this has not yet been

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[1] *Abbreviations used:* PDI, protein disulfide isomerase; ANOVA, analysis of variance; eGFP, enhanced green fluorescent protein; cPABP, chloroplast polyadenylate-binding protein.

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investigated. Moreover, the consequences of PDI modulation of NADPH oxidase are potentially quite important, considering the known PDI effects in processes such as cellular response to hypoxia [15] and protein misfolding [16], protein secretion [11], cell adhesion [17], coagulation [18] and microorganism invasion [19,20]. PDI might be involved in those processes through its versatile catalytic effects involving its essential thiol redox and isomerase activities, as well as through its thiol-independent chaperone activity [10]. Particularly, the role of the two PDI catalytic dithiol thioredoxin motifs in its interaction with NADPH oxidase is unknown. We hypothesized that PDI might regulate the expression of catalytic Nox subunits, in addition to NADPH oxidase activity, in a way that might depend on PDI catalytic thiol groups. In addition, we sought to investigate whether exposure to nitric oxide could modulate NADPH oxidase activity and expression in a way dependent on its known reaction with PDI thiols, which are known to promote inhibition of isomerase activity [21,16]. Our aim was to investigate the effects of PDI loss- or gain-of-function experiments on activity and mRNA expression of Nox1 and Nox4 subunits in VSMC. In addition, we compared the effects of PDI overexpressed in its wild-type configuration with those after mutation of all four active site thiols [16]. Moreover, we investigated effects of GSNO exposure on activity and expression of NADPH oxidase in the presence or not of PDI overexpression.

Materials and methods

Materials and cell culture

Antibodies against PDI were purchased from Stressgen (Ann Arbor, MI) and Affinity Bioreagents (Golden, CO); Nox1 and Nox4 from AbCam (Cambridge, MA); β-actin from Sigma (St. Louis, MO); secondary antibodies conjugated with horseradish peroxidase from Calbiochem (San Diego, CA, USA). Protease inhibitors were from Calbiochem (La Jolla, CA); GSNO from Calbiochem (San Diego, CA, USA); dihydroethidium, AmplexRed reagent and Lipofectamine 2000 from Invitrogen (Carlsbad, CA). All other reagents were from Sigma (St. Louis, MO). Rabbit aortic smooth muscle cells were obtained from a previously established selection-immortalized line [8] and maintained in growth medium (F12 + 10% fetal bovine serum + streptomycin 100 μM + penicillin 100 U/mL).

VSMC transfection

For cDNA transient transfection, VSMC (8×10^5 cells/well) were cultured for 24 h and incubated with 5 μg of rat wild-type or mutated (C36, 39, 383, 386S) PDI cDNA (pCR 3.1, kindly provided by Dr. Tomohiro Nakamura, from Burnham Institute for Medical Research, CA) and Lipofectamine 2000 (5 μL, Invitrogen) in growth medium without serum and antibiotics for 8 h. Transfection medium was replaced by growth medium without serum for further experiments, which were performed 18–24 h later. Other plasmids employed were: pCDNA3 (empty vector control) and pCMV-eGFP (kindly provided by Dr. José Xavier Neto, from Heart Institute, Brazil). For siRNA transfection, VSMC (1.2×10^5 cells/well) were cultured in growth medium without serum and antibiotics and incubated for 6 h with rabbit PDI siRNA oligonucleotides (50 nM final concentration) and Lipofectamine 2000 (5 μL). PDI siRNA sequence was obtained with BLOCK-iT™ RNAi Designer, from Invitrogen (GAGGUGGCCUUUGACGAGAAGAAGA). This PDI siRNA concentration was able to effectively silence at least 70% of total PDI protein assessed by Western blot in preliminary dose–response studies. Control siRNA was Stealth™ RNAi Negative Control Duplexes (medium GC), from Invitrogen. Transfection medium was replaced by growth medium for 48 h and then switched to growth medium without serum for additional 18–24 h, after which experiments were performed.

Cellular ROS measurement

Cellular H_2O_2 production was measured by VSMC incubation with AmplexRed reagent (0.1 mM) and horseradish peroxidase (1 U/mL) in Krebs buffer (in mM: $CaCl_2$ 0.5; $MgSO_4$ 1.2; KCl 4.9; KH_2PO_4 5.7; NaCl 145 mM; Na_2HPO_4 5.7; glucose 5.5, pH 7.4) containing DTPA (0.1 mM) for 30 min at 37 °C. Cells in suspension were separated into two wells, with one well serving as an internal control with added catalase (200 U/mL). Quantification was calculated from the difference between fluorescence without and with catalase. Fluorescence was followed in a microplate reader (excitation/emission wavelengths 480/560 nm) in a spectrofluorometer (SpectraMax M5, Molecular Devices). Cellular superoxide production was measured by 2-hydroxyethidium quantification by HPLC, after acetonitrile extraction of VSMC incubated with dihydroethidium (50 μM) for 30 min in Krebs buffer with DTPA as described above [22].

NADPH oxidase activity assay

VSMC were disrupted by sonication in buffer containing Tris 50 mM, pH 7.4, EDTA 0.1 mM, EGTA 0.1 mM, and protease inhibitors (aprotinin 10 μg/mL, leupeptin 10 μg/mL and PMSF 1 mM), and centrifuged (18,000*g*, 15 min). After supernatant centrifugation (100,000*g*, 1 h), the obtained pellet (VSMC membrane fraction) was resuspended in the same buffer. For superoxide-derived NADPH oxidase, membrane homogenates (15 μg protein) were incubated with dihydroethidium (10 μM) in phosphate buffer (50 mM, pH 7.4) with DTPA 0.1 mM in the presence of NADPH (50 μM) and DNA (1.25 μg/mL) for 30 min at 37 °C in the dark [22]. For H_2O_2-derived NADPH oxidase, membrane homogenates (15 μg protein) were incubated with AmplexRed reagent (0.25 mM) and horseradish peroxidase (10 units/mL) in phosphate buffer/DTPA in the presence of NADPH (250 μM) for 30 min at 37 °C in the dark. Fluorescence was followed in a microplate reader (excitation/emission wavelengths for dihydroethidium: 490/590 nm; AmplexRed reagent: 480/560 nm, respectively) in a spectrofluorometer (SpectraMax M5, Molecular Devices).

Quantitative PCR

RNA was isolated with RNA SpinMini RNA isolation kit (GE Healthcare) and was converted to cDNA by incubation of 3 μg mRNA, 25 ng/μL OligodT(12-18), 500 μM (each) dNTP, 5 μM dithiothreitol and SuperScript II (Invitrogen) at 42 °C for 50 min. Quantitative PCR was performed with 150 ng of cDNA and Sybr Mastermix (Invitrogen) and was analyzed with Rotor-Gene 6000 Software (Corbett Research). Forward primers designed according to rabbit sequences were: Nox1 – CATCATGGAAGGAAGGAGA; Nox4 – CCACAGACTTGGCTTTGGAT; GAPDH – TCACCATCTTCCAG GAGCGA. Rabbit Nox sequences were kindly provided by Dr. Bernard Lassegue (Emory University, EUA).

Western blot

VSMC homogenates were obtained by cell lysis in RIPA buffer (Tris 20 mM, pH 8, NaCl 137 mM, NP-40 1% and glycerol 10%), with protease inhibitors (aprotinin 10 μg/mL, leupeptin 10 μg/mL, PMSF 1 mM). After 20 min on ice, samples were centrifuged (1000*g*, 10 min) and supernatants analyzed in SDS–PAGE. After protein transfer to nitrocellulose membrane, membranes were blocked with non-fat milk (5%), blotted with primary antibodies overnight, blotted with secondary antibodies conjugated with horseradish peroxi-