A possible biochemical link between NADPH oxidase (Nox) 1 redox-signalling and ERp72

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Emerging evidence indicates that Nox (NADPH oxidase) 1-generated ROS (reactive oxygen species) play critical regulatory roles in various cellular processes, yet little is known of direct targets for the oxidase. In the present study we show that one of the proteins selectively oxidized in response to Nox1-generated ROS was ERp72 (endoplasmic reticulum protein 72 kDa) with TRX (thioredoxin) homology domains. Oxidation of ERp72 by Nox1 resulted in an inhibition of its reductase activity. EGF treatment of cells stimulated the Nox1 activity and the activated Nox1 subsequently mediated EGF-induced suppression of the ERp72 reductase activity. Co-immunoprecipitation, GST (glutathione transferase) pulldown assays and mutational analysis, indicated that Nox1 associates with ERp72, which involves its N-terminus encompassing a Ca\(^{2+}\)-binding site and the first TRX-like motif. Furthermore, confocal microscopy showed co-localization between Nox1 and ERp72 at the plasma membrane. These results suggest that Nox1 functionally associates with ERp72, regulating redox-sensitive signalling pathways in a cellular context.

Key words: endoplasmic reticulum protein 72 kDa (ERp72), NADPH oxidase (Nox1), reactive oxygen species, redox signalling.

INTRODUCTION

ROS (reactive oxygen species), notably superoxide anion and H\(_2\)O\(_2\), are generated in response to a variety of extracellular stimuli including cytokines, peptide growth factors and hormones and play roles as signalling molecules in physiological processes such as host defence, regulation of vascularization, oxygen sensing, apoptosis and cell transformation [1]. The recent discovery of ROS-generating enzymes responsible for these processes revealed the existence of the Nox (NADPH oxidase) family, homologues of phytochrome gp91phox (Nox1-5, Duox1 and Duox2) [2]. The members of the Nox family are expressed in various tissues and are likely to display distinctive, specific physiological functions. For example, it has been known that EGFR (epidermal growth factor) stimulates the activity of Nox1 to produce superoxide and generated superoxide is rapidly dismutated to H\(_2\)O\(_2\), leading to EGF-induced physiological responses [3,4].

In general, Nox proteins, having binding sites for haem, FAD and NADPH, catalyse the transfer of an electron from molecular oxygen to generate superoxide anion which is enzymatically dismutated to H\(_2\)O\(_2\) [5]. H\(_2\)O\(_2\) presumably propagates signalling responses by modulating hypothetical effector molecules. Although many studies have been aimed at dissecting the Nox-mediated signalling pathways, little is known about the downstream targets for Nox-generated ROS. We previously demonstrated oxidative inactivation of the low-molecular-mass protein tyrosine phosphatase by Nox1, a critical event that mediates oncogenic Ras-induced disruption of stress fibres and focal adhesions by down-regulating Rho [6,7]. With regard to the importance of this target issue, we decided to identify and characterize other molecular target proteins for Nox1-derived ROS.

In the present study, we identified ERp72 [ER (endoplasmic reticulum) protein 72 kDa] as one of the possible downstream targets for Nox1-generated ROS. Nox1 spatially associated with ERp72 and inhibited the in vitro reductase activity of ERp72. Furthermore, Nox1 mediated EGF-induced suppression of the ERp72 reductase activity. Thus the present study suggests that Nox1-dependent redox signalling pathways biochemically link to ERp72.

ERp72 belongs to the family of PDI (protein disulfide isomerases) which catalyse disulfide bond formation, reduction or isomerization of newly synthesized proteins in the ER lumen [8]. They also function as molecular chaperones that are critical elements in a quality-control system for the correct protein folding in response to ER stress such as disruption of Ca\(^{2+}\) homoeostasis and inhibition of protein glycosylation [9]. These biochemical properties stem from oxidation and reduction of cysteine-thiols in TRX (thioredoxin) domains of the PDI family [10]. Despite bearing the ER retention sequence KDEL, some PDIs are located in a non-ER compartment such as the cell surface and the cytosol, and the functional roles under such an environment are much less known [8]. In this respect, it is noteworthy that a possible biochemical link between Nox1 and ERp72 at the plasma membrane was detected in the present study. The biological significance of the finding is discussed in relationship with the ERp72 functions.

Abbreviations used: ASK1, apoptosis signal-regulating kinase 1; BIAM, N-(iodoacetyl) ethylenediamine; DPI, diphenyleneiodonium chloride; DTT, dithiothreitol; EGFR, epidermal growth factor; ER, endoplasmic reticulum; ERp72, ER protein 72 kDa; GST, glutathione transferase; HBSS, Hank’s balanced salt solution; HE, hydroethidine; HP, hypotonic; HRP, horseradish peroxidase; 5-IAF, 5-iodoacetamide fluorescein; K-NRK, Kirsten-normal rat kidney; K-Ras-NRK, Kirsten-Ras-transformed NRK; NEM, N-ethylmaleimide; Nox, NADPH oxidase; PDI, protein disulfide isomerase; ROS, reactive oxygen species; siRNA, small interfering RNA; TRX, thioredoxin.

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Figure 1 Identification of cellular proteins oxidized in response to Nox1-generated ROS

(A) Cos-1 cells (5 × 10^6) were co-transfected with Nox1, NOXO1 and NOXA1, or control vectors. At 48 h later, cells were harvested and lysed in lysis buffer containing 5-IAF. Labelled proteins were subjected to SDS/PAGE and immunoblot (IB) analysis with antibodies to fluorescein. Protein loading was monitored by Coomassie Brilliant Blue (CBB) staining. The molecular mass in kDa is indicated on the left-hand side. (B) Cos-1 cells were transfected with control vectors or Nox1, NOXO1 and NOXA1 as in (A). At 48 h later, superoxide production was determined as described in the Materials and methods section. The elution of 2-OH-E^+ and E^+ peaks is shown with their retention times. The retention time of the standards is shown in Supplementary Figure S1(A) at http://www.BiochemJ.org/bj/416/bj4160055add.htm. The HPLC peak intensity was calibrated as described in the Materials and methods section. Values represent means ± S.D. in three independent experiments; *P < 0.01 compared with control vectors. (C) NRK and K-NRK cells (2 × 10^6) were lysed in lysis buffer supplemented with BIAM and labelled as described in the Materials and methods section. Labelled proteins were separated by SDS/PAGE and detected by immunoblot (IB) analysis with HRP-conjugated streptavidin. Protein loading was monitored by Coomassie Brilliant Blue (CBB) staining. The molecular mass in kDa is indicated. (D) Affinity purification of BIAM-labelled proteins. K-NRK cells (10^8) were lysed in lysis buffer and labelled with BIAM as described in the Materials and methods section. Lysates were loaded on to a streptavidin–agarose column. Bound proteins were eluted and analysed by SDS/PAGE, followed by immunoblotting (IB) with HRP-conjugated streptavidin. The molecular mass in kDa is indicated. CBB, Coomassie Brilliant Blue.

MATERIALS AND METHODS

Cell culture and materials

Human colon cancer Caco-2 cells and Cos-1 cells were obtained from American Type Culture Collection and maintained as described previously [11]. 5-IAF (5-iodoacetamide fluorescein) and BIAM [N’(iodoacetyl) ethylenediamine] were purchased from Molecular Probes, protein G-Sepharose and protein A-Sepharose were from Invitrogen, and DPI (diphenyleneiodonium chloride) were from Calbiochem. HRP (horseradish peroxidase)-conjugated streptavidin and streptavidin-conjugated agarose were obtained from Pierce. EGF, mouse anti-Flag antibodies and NEM (N-ethylmaleimide) was from Sigma, and rabbit anti-ERp72 antibodies were from Stressgen. Mouse anti-Nox1 antibodies were provided by Dia Dexus. HE (hydroethidine) was obtained from Polysciences, and xanthine and xanthine oxidase were purchased from Nacalai Tesque.

Construction of plasmids

pCMV2-Flag-ERp72 has been described previously [12]. Rat ERp72 cDNA was subcloned into pGEX-2T at BamHI and Smal sites. pcDNA3.0-Nox1, pEFBOS-NOXO1 and pEFBOS-NOXA1 have been described previously [13].

Labelling with 5-IAF or BIAM

The labelling procedure in Figures 1 and 3(A) is based on previously published methods [14,15] with a slight modification under anaerobic conditions. Cells were washed with PBS and lysed in lysis buffer [25 mM Mops/NaOH (pH 6.5), 10% glycerol, 1% Triton X-100, 100 mM NaCl, 1 μg/ml catalase, 1 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml aprotinin] supplemented with 12 μM 5-IAF at 4°C for 20 min. Lysates were incubated at 37°C for 5 min. The labelling reaction was terminated by the addition of SDS sample buffer. BIAM labelling was performed as described above except for the addition of 20 μM BIAM to the lysis buffer. Labelled proteins were subjected to SDS/PAGE, followed by detection with HRP-conjugated streptavidin and ECL® Plus (GE Healthcare).

An alternative labelling method in Figures 3(B) and 3(C) was performed according to a method described previously [16]. Cells were transfected with Flag–ERp72 and harvested in a lysis buffer [0.1 M sodium phosphate (pH 7.0), 5 mM EDTA,
5 mM EGTA, 1% Nonidet P40 and 1 mM PMSF) containing 20 mM NEM, and sulfhydryl groups of free cysteine residues were blocked through alkylation by incubating for 30 min at 4°C. Lysates were dialysed against 500 ml of PBS for 8 h. The reduction reaction was then achieved by adding 20 mM DTT (dithiothreitol) for 30 min, followed by dialysis against PBS overnight. Samples were labelled with 20 μM BIAM for 1 h at 4°C. Flag–ERp72 was immunoprecipitated by anti-Flag antibodies, and the immunoprecipitates were analysed by SDS/PAGE, followed by HRP–streptavidin staining.

Affinity purification of BIAM-labelled proteins and MS analysis

Chromatography was performed as described previously [15]. K-NRK (Kirstein-Ras-normal rat kidney) cells (106 cells) were labelled in lysis buffer containing 20 μM BIAM as in Figure 1 and the labelling was terminated by the addition of 20 mM 2-mercaptoethanol. After dialysis against PBS for 3 h, lysates were loaded on to a streptavidin–agarose column (1 ml). Bound proteins were eluted with 8 M guanidine/HCl and dialysed into a previously published protocol [17]. Affinity-purified proteins were digested by trypsin and the peptides were fractionated by HPLC with a C18 column (Michron Bio Resources). Peptide fragments were subjected to LC-ESI-MS/MS (liquid chromatography-electrospray ionization tandem MS) analysis by utilizing Paradigm MS-4 (Michron Bio Resources) and LCQ advantage (Thermo Electron).

Transfection

Cells were transfected with expression vectors using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol.

GST (glutathione transferase) pulldown assay and interaction study

Cos-1 cells were co-transfected with pcDNA3.0-Nox1 (10 μg), pEFBOS-NOXO1 (3 μg) and pEFBOS-NOXA1 (3 μg) and lysed in RIPA buffer [50 mM Tris/HCl (pH 7.2), 1% Nonidet P40, 0.1% sodium deoxycholate, 150 mM NaCl, 4 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml aprotinin] [12]. Lysates were incubated with fusion protein-coupled resins for 3 h at 4°C and bound proteins were analysed by SDS/PAGE, followed by immunoblotting with monoclonal anti-Nox1 antibodies.

ERp72 fragments, 1–105, 102–643 and 1–548 amino acid residues were inserted into pGEX-6P-1 at BamHI/Smal, pGEX-6P-1 at BamHI/Xhol and pGEX-2T at BamHI/EcoRI respectively. Lysates were prepared from Nox1-transfected Cos-1 cells (106) and incubated with GST-fusion proteins (3 μg)-coupled resins. Bound proteins were analysed by immunoblotting as described above.

Immunoblotting and immunoprecipitation

Cells were homogenized in RIPA buffer. Lysates were processed to immunoblotting or immunoprecipitation as described. Proteins bound to the resins were separated by SDS/PAGE and probed by immunoblotting with various antibodies.

Immunohistochemistry

Cells were plated on glass coverslips, fixed in 3.7% (w/v) paraformaldehyde, and permeabilized in 0.2% Triton X-100 as described [7]. The cells were stained with the various antibodies indicated and observed under a Zeiss confocal microscope (LSM510; Carl Zeiss).

Subcellular fractionation

Plasma membrane fractions were isolated as described previously [18]. Caco-2 cells (2 × 105) were harvested and resuspended in 1 ml of HP (hypotonic) buffer [5 mM Tris/HCl (pH 7.5) and 1 mM PMSF] and homogenized on ice. The homogenates were spun at 500 g for 5 min and the supernatants were centrifuged at 40 000 rev./min. (SW50.1 rotor; Beckman) for 20 min. The pellets were resuspended in 4 ml of HP buffer containing 1.42 M sucrose and the samples were overlayed with 0.8 ml of 0.25 M sucrose in HP buffer and centrifuged at 40 000 rev./min. (SW50.1 rotor; Beckman) for 1 h. Plasma membranes at the interface were collected and spun at 40 000 rev./min. (RP80 rotor; Hitachi) for 20 min. The pellets were solubilized in 0.5 ml of RIPA buffer and saved as plasma membrane extracts.

HPLC/fluorescence assay using HE for superoxide generation

The HPLC/fluorescence assay was performed as described previously [19]. After transfection or chemical-treatment, cells (1 × 10 cm dish) were washed with HBSS (Hank’s balanced salt solution) and incubated with HBSS containing 10 μM HE for 20 min. Cells were lysed in 250 μl of PBS containing 0.1% Triton X-100 and extracted with 500 μl of 1-butanol. The samples in the butanol phase were dried, dissolved in 40 μl of water and loaded on to a C18 column. HE, E2 and HE/O2•−-derived product were eluted by a linear gradient of acetonitrile (10–70%) in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. The elution was monitored by a fluorescence detector with excitation and emission at 510 nm and 595 nm respectively. Sample amounts were normalized by the protein concentration of cell lysates (Bio-Rad). HPLC peak intensity was calibrated based on the relationships between the amount of 2-OH-E2 and fluorescence intensity (Supplementary Figure S1D at http://www.BiochemJ.org/bj/416/bj4160055add.htm).

Insulin reduction assay

The insulin reduction assay was performed as described previously [20]. The validity of the assay method was confirmed in control experiments using purified PDI proteins (Sigma). Cells (5 × 106) were co-transfected with pCMV2-Flag-ERp72 (1 μg), pcDNA3.0-Nox1 (4 μg), pEFBOS-NOXO1 (1 μg) and pEFBOS-NOXA1 (1 μg), lysed in 0.5 ml of RIPA buffer under anerobic conditions. Cell lysates were immunoprecipitated with anti-Flag antibodies and protein G–Sepharose (20 μl). The resins were washed three times with RIPA buffer and once with insulin reduction assay buffer [100 mM potassium acetate (pH 7.5), 2 mM EDTA and 10 μM DTT]. A 10 μl aliquot of Flag–ERp72 immobilized resins (≈100 ng of protein) was mixed with 110 μl of assay buffer containing 1 mg/ml insulin. The reaction was initiated by adding 0.2 mM DTT and proceeded at 37°C for 20 min. Then, the reaction mixtures were spun down at 500 g for 1 min and insulin turbidity in the supernatants was determined by measuring the absorbancy at 650 nm.

Statistical analysis

The statistical analysis was performed with a Student’s t test. For multiple treatment groups, a factorial ANOVA followed by Bonferroni’s t test was applied. Differences with values P < 0.05 were considered to be statistically significant.
RESULTS

Identification of proteins oxidized in response to Nox1-mediated ROS production

It is conceivable that Nox1-generated superoxide is rapidly converted into H$_2$O$_2$ and that H$_2$O$_2$ subsequently oxidizes cysteine residues of target proteins. H$_2$O$_2$ can mildly oxidize thiols of cysteine residues, yielding sulfenic acid (Cys-SOH) or disulfide, both of which are reduced to cysteine residues by cellular reductants. If these cysteine residues were located near the structurally critical areas including the active centre of proteins, the modification would have the potential to regulate the function of proteins. This oxidation reaction is reversible and the number of H$_2$O$_2$-sensitive cysteine residues would be relatively limited [14]. These biochemical properties allow the H$_2$O$_2$-induced oxidation to elicit flexible signalling responses to extracellular stimuli and play a specific role in the regulation of cellular metabolism. Labelling of cysteine residues with 5-IAF or BIAM under acidic conditions has been developed to detect proteins with reactive cysteine residues and has proved quite useful in identifying H$_2$O$_2$-sensitive proteins involved in receptor-mediated signal transduction including PTP1B (protein tyrosine phosphatase-1B) [14,15].

To identify the proteins with H$_2$O$_2$-sensitive cysteine residues, Cos-1 cells were transfected with Nox1 and its adaptor proteins NOXO1 and NOXA1 and lysed in a pH 5.5–7.0 lysis buffer containing 5-IAF. Labelled proteins were detected by immunoblotting with antibodies to fluorescein. 95, 72, 58 and 52 kDa proteins were predominantly labelled in control vector-transfected cells and the extent of labelling of the proteins was decreased when Nox1 and adaptor proteins were overexpressed (Figure 1A). The ROS assay confirmed that the level of 2-OH-E$_2^-$, the product of reaction of HE with O$_2^•^-$ was increased upon transfection of Nox1 with its adaptor proteins, suggesting an increase in Nox1-derived superoxide production (Figure 1B). The results indicate that cysteine residues of these proteins are susceptible to ROS generated by Nox1 enzymes.

Our previous study showed that Nox1 expression is up-regulated in K-Ras-NRK (Kirstein-Ras-transformed NRK) cells compared with NRK cells [6], raising the possibility that augmented generation of Nox1-derived ROS leads to oxidation of cellular proteins similar to those in Cos-1 cells overexpressing Nox1. As expected, labelling experiments with BIAM show that labelling efficiency of the 95, 72 and 58 kDa proteins was reduced in K-Ras-NRK cells as compared with NRK cells (Figure 1C) and DPI, a Nox1 inhibitor, treatment restored labelling of proteins (results not shown). To characterize the nature of labelled proteins, we affinity-purified BIAM-labelled proteins from K-Ras-NRK cells by using streptavidin–agarose. K-Ras-NRK cells were lysed in acidic lysis buffer containing BIAM, and labelled proteins were fractionated with the affinity resins. SDS/PAGE and streptavidin blot analysis show that the 95, 72 and 58 kDa proteins were partially purified (Figure 1D). These protein bands were processed for MS analysis.

With regards to the 72 kDa protein, its trypsin digests were fractionated by HPLC and their primary structures were obtained by LC-MS/MS. Amino acid sequences of two peptide peaks, peptides #1 and #2 in the HPLC elution profile were found to be identical with residues 271–286 and 369–384 of ERp72 respectively [20] (Figure 2). Immunoblotting analysis also demonstrated that the 72 kDa protein band was recognized by antibodies against ERp72 (results not shown). ERp72 belongs to the PDI family and has three TRX-like WCGHC motifs responsible for redox activity [21]. The protein, like PDI, is considered to act as a reductase, oxidase or isomerase by catalysing the formation and isomerization of disulfide bonds in target proteins [9,22].

ERp72 is oxidized by Nox1-generated ROS

To investigate the functional relationships between ERp72 and Nox1 redox-signalling, we first confirmed that ERp72 was oxidized by Nox1-generated ROS. ERp72 was introduced into Cos-1 cells together with empty vectors or vectors encoding Nox1, NOXO1 and NOXA1, lysates were incubated with BIAM, and the extent of BIAM labelling of ERp72 was examined. The level of labelled ERp72 was decreased in Nox1-transfected cells compared with control-vector-transfected cells (Figure 3A), which can be explained by the fact that redox-sensitive thiols of cysteine residues react with BIAM. Alternatively, the active cysteine residues can be detected by the method in which cysteine residues oxidized by ROS are protected from post-labelling alkylation with NEM and reacted with the iodoacetic-derivative labelling reagents following reduction with DTT [16]. Using this approach, we found that Nox1 transfection promoted BIAM labelling of ERp72 (Figure 3B), and that apocynin or DPI treatment blocked the labelling (Figure 3C). Taken together, these results are consistent with the notion that Nox1-generated ROS oxidized redox-sensitive cysteine residues of ERp72.

Interaction of ERp72 with Nox1

To explore whether Nox1 interacts with ERp72, GST–ERp72 fusion proteins were created (Figure 4A). Cos-1 cells were transfected with Nox1, NOXO1 and NOXA1. Cell lysates were incubated with GST–ERp72-coupled resins. Nox1 proteins were co-precipitated with GST–ERp72, but not GST alone (Figure 4B). It should be noted that association of Nox1 with ERp72 did not require the Nox1 adaptor proteins, NOXO1 and NOXA1, because...
Figure 3  Nox1 suppresses BIAM labelling of ERp72

(A–C) Cos-1 cells (5 × 10⁶) were transfected with vectors encoding ERp72, Nox1, NOXO1 and NOXA1, or control vectors and lysed 48 h later. (A) Lysates were labelled with BIAM as described in the Materials and methods section. Labelled proteins were immunoprecipitated with anti-ERp72 antibodies and the immunoprecipitates were probed with HRP-conjugated streptavidin. *P < 0.01 compared with control vectors. (B) Labelling of Flag–ERp72 proteins, following alkylation and reduction, was performed as described in the Materials and methods section. *P < 0.01 compared with control vectors. (C) Transfected cells were further treated with 10 μM DPI or 5 μM apocynin for 1 h. Assay of ERp72-labelling was performed as described in (B). *P < 0.05, †P < 0.01 and ‡P < 0.05 compared with control vectors. (A–C) histograms show the densitometric intensity (means ± S.D.) of labelled ERp72 from three independent experiments. The expression of transfected proteins was verified by immunoblotting.

Next, the domain of ERp72 responsible for the interaction with Nox1 was determined by deletion mutation analysis. ERp72 is composed of a putative Ca²⁺-binding region and three TRX homology domains (α, a and a’) containing WCGHC sequences [10]. To determine the Nox1-binding region of ERp72, various deletion constructs fused to GST were created (Figure 5A), and Nox1 bound to the GST fusion proteins was detected by immunoblotting following incubation with lysates from Nox1-overexpressing Cos-1 cells. The wild-type ERp72, 1–105 and 1–548 mutants bound to Nox1, whereas the C-terminal half-fragment (102–643) did not (Figures 5A and 5B). The results indicate that ERp72 specifically interacted with Nox1 through its N-terminal domain containing the putative Ca²⁺-binding region and the α region.

We next determined whether ERp72 was intracellularly co-localized with Nox1. Cos-1 cells were transfected with Nox1 and subjected to double immunostaining with antibodies against ERp72 and Nox1. The specificity of mouse monoclonal anti-Nox1 antibodies was confirmed by immunoblotting analysis (results not shown). As expected, most of the ERp72 was distributed in the area of perinuclear ER and the cytosol, but some of the ERp72 was located at the plasma membrane and co-localized with ectopic Nox1 in 36.9 ± 4.3% (n = 3) of Cos-1 cells [Figure 6A].

Figure 4  Nox1 interacts with ERp72

(A) GST and GST–ERp72wt (wt, wild-type) were prepared, immobilized to GST–Sepharose (Amersham Pharmacia) beads, and analysed by SDS/PAGE and Coomassie Brilliant Blue staining. (B) Cos-1 cells (2 × 10⁶) were transfected with vectors encoding Nox1, NOXO1 and NOXA1, or control vectors. Lysates were incubated with GST fusion protein-coupled resins. Nox1 bound to the resins was detected by immunoblotting with anti-Nox1 antibodies. (C) Cos-1 cells (5 × 10⁶) were co-transfected with vectors encoding ERp72 and Nox1 or control vectors. Lysates were subjected to immunoprecipitation with anti-ERp72 antibodies, followed by immunoblotting with anti-Nox1 antibodies. ERp72 in the immunoprecipitates was detected by immunoblotting with anti-ERp72 antibodies. A representative in four experiments is shown.

a similar Nox1 co-precipitation was detected with lysates from cells overexpressing Nox1 alone. To further assess the interaction between ERp72 and Nox1, co-immunoprecipitation experiments were performed. Cos-1 cells were co-transfected with Nox1 and ERp72 expression plasmids. ERp72 immunoprecipitation yielded co-precipitation of Nox1 (Figure 4C). These results suggest that ERp72 associates with Nox1. In control experiments, treatment of GST–ERp72 proteins with 2 mM H₂O₂ for 10 min at 37°C, which causes oxidation of reactive cysteine residues [14], did not influence ERp72 binding to Nox1 (results not shown), making it unlikely that oxidation of ERp72 by Nox1 alters its binding to Nox1.
cells is consistent with these previous observations. is partially located at the plasma membrane in Cos-1 and Caco-2 cytosol and nuclei [9]. The finding of the present study that ERp72 is found in non-ER compartments such as the cell surface, plasma membranes, the mitochondria (mt1–105, mt1–548, mt1–548). Three TRX homology domains a, a and a’ and a putative Ca2+ binding region (c) are indicated. The choice of construct lengths was based on solubility advantage over proper domain constructs and we made mt1–105 and mt1–548 instead of 1–170 and 1–510 respectively. (B) GST–ERp72 wt (wt, wild-type) and mutant protein-coupled resins were incubated with lysates prepared from Nox1-overexpressing Cos-1 cells. Nox1 proteins bound to the resins were analysed by immunoblotting (IB) with anti-Nox1 antibodies. Direct immunoblots of lysates are shown as controls. A representative of three separate experiments is shown. GST–ERp72 wt and mutant fusion proteins were prepared and analysed by SDS/PAGE, followed by Coomassie Brilliant Blue (CBB) staining.

Figure 5 Analysis of the Nox1-binding domain of ERp72

(A) A schematic diagram of GST–ERp72 full length and deletion mutant constructs (mt1–105, mt1–548 and mt1–643). Three TRX homology domains a, a and a’ and a putative Ca2+ binding region (c) are indicated. The choice of construct lengths was based on solubility advantage over proper domain constructs and we made mt1–105 and mt1–548 instead of 1–170 and 1–510 respectively. (B) GST–ERp72 wt (wt, wild-type) and mutant protein-coupled resins were incubated with lysates prepared from Nox1-overexpressing Cos-1 cells. Nox1 proteins bound to the resins were analysed by immunoblotting (IB) with anti-Nox1 antibodies. Direct immunoblots of lysates are shown as controls. A representative of three separate experiments is shown. GST–ERp72 wt and mutant fusion proteins were prepared and analysed by SDS/PAGE, followed by Coomassie Brilliant Blue (CBB) staining.

(E–C). Similarly, endogenous ERp72 and Nox1 proteins were co-localized at the plasma membrane in 52.4 ± 2.3% (n = 3) of Caco-2 cells [Figure 6A (d–f)]. To biochemically analyse the subcellular distribution of Nox1 and ERp72, microsome-free plasma membranes were purified from Nox1-transfected Cos-1 cells using sucrose-density-gradient ultracentrifugation and subjected to immunoblotting. The results indicate that both Nox1 and ERp72 were detected in the plasma membrane fraction (Figure 6B). Although ERp72 possesses an ER retention signal sequence (KEEL) at the C-terminus and is enriched in the ER, there is mounting evidence that ERp72 is found in non-ER compartments such as the cell surface, plasma membranes, the cytosol and nuclei [9]. The finding of the present study that ERp72 is partially located at the plasma membrane in Cos-1 and Caco-2 cells is consistent with these previous observations.

Oxidation of ERp72 by Nox1 inhibits its reductase activity

The functional consequence of Nox1-catalysed oxidation of ERp72 remains to be investigated. ERp72 protein is known to catalyse the reduction of disulfide bonds in proteins such as insulin [23]. Using an insulin reduction assay, we determined whether oxidation of cysteine residues in TRX-like domains of ERp72 by Nox1 affected its reductase activity. We were unable to carry out in vitro reconstitution experiments using GST–ERp72 proteins and bacterially made recombinant Nox1 proteins because the recombinant Nox1 proteins were not solubilized in physiological buffers (W. Chen and T. Kamata, unpublished work). Instead, Cos-1 cells were transfected with Flag–ERp72, Nox1, NOXO1 and NOXA1, which allows the modulation of ERp72 activity by oxidation in vivo. Cell lysates were immunoprecipitated with anti-Flag antibodies and ERp72 immunoprecipitates were tested for the ability to reduce insulin. Overexpression of Nox1 and its adaptors decreased the insulin reduction activity of ERp72 compared with vector controls and apocynin restored the activity, suggesting that Nox1-derived ROS, but not direct binding of Nox1 to ERp72, was essential for ERp72 inhibition (Figure 7A). Possibly, Nox1 maintains ERp72 in an oxidized state and thereby attenuates the reductase activity of ERp72.

EGF signalling inhibits the reductase activity of ERp72 through Nox1

Nox1 has been reported to couple to the EGF receptor system and mediate EGF-stimulated production of ROS [4]. We therefore reasoned that if EGF is a potent stimulator of Nox1, EGF receptor activation should trigger Nox1-dependent suppression of ERp72 reductase activity. To examine the effect of EGF on ERp72 action, we first confirmed whether EGF induces Nox1-mediated ROS production in Caco-2 cells. Caco-2 cells were preloaded with HE and subjected to a superoxide generation assay. In agreement with a previous report [4], EGF treatment rapidly induced superoxide generation (Figure 7B). By contrast, DPI and apocynin treatment (Figure 7B) or silencing of Nox1 by siRNAs (small interfering RNAs) (Figure 7C) resulted in a decrease in ROS production in response to EGF stimulation, indicating that EGF transmits an activation signal to Nox1. In control experiments, rotenone, a mitochondrial oxidase inhibitor...
Figure 7 Nox1 regulates the reductase activity of ERp72

(A) Cos-1 cells were transfected with vectors encoding Flag-ERp72, Nox1, NOXO1 and NOXA, or control vectors, and treated with 5 μM apocynin. ERp72 was immunoprecipitated with anti-Flag antibodies and the immunocomplexes were subjected to insulin reduction assay or immunoblotting with anti-Flag antibodies. The reductase activity is normalized to that in cells transfected with control vectors (the average absorbancy is 0.1). Values represent means ± S.D. in three separate experiments. *P < 0.01 compared with control. **P < 0.01 compared with Apocynin(−)/Nox1/NOXO1/NOXA1-transfected cells. (B and C) Caco-2 cells (10⁵) were serum-starved, treated with or without 10 μM DPI, 5 μM apocynin or 50 μM rotenone for 1 h prior to HE loading and EGF (200 ng/ml) treatment for 5 min (B). Alternatively, cells were transfected with Nox1 siRNAs or scrambled siRNAs for 48 h, serum-starved, loaded with HE and treated with EGF (200 ng/ml) for 5 min (C). Intracellular superoxide generation was measured based on 2-OH-E+ fluorescence intensity. Fluorescence intensity was calibrated as described in the Materials and methods section. Values are means ± S.D. of triplicate experiments. Silencing of endogenous Nox1 by Nox1 siRNAs was determined as in (E) (results not shown). *P < 0.05 and **P < 0.01 compared with EGF-treated cells. †P < 0.05 compared with scrambled siRNA. (D and E) Caco-2 cells (2 × 10⁶) were co-transfected with Flag-ERp72, NOXO1 and NOXA1, serum-starved and treated with or without DPI (5 μM) for 1 h prior to EGF (200 ng/ml) stimulation for 10 min (D). Alternatively, cells were transfected with Nox1 siRNAs or scrambled siRNAs, serum-starved and stimulated with EGF as described above (E). Lysates were prepared, Flag-ERp72 was immunoprecipitated, and ERp72 immunoprecipitates were subjected to an insulin reduction assay. The reductase activity is normalized to that in untreated cells (D) or scrambled siRNA-transfected cells (E) as in (A). Values are means ± S.D. in three separate experiments. *P < 0.01 compared with untreated cells. **P < 0.05 compared with cells treated with DPI(−)/EGF(+). *P < 0.05 compared with scrambled siRNA (E). ERp72 in immunoprecipitates was monitored by immunoblotting. Suppression of endogenous Nox1 by Nox1 siRNAs was examined by immunoblotting with anti-Nox1 antibodies.

DISCUSSION

It is becoming clear that Nox1-mediated redox regulation is critical for multiple cellular processes including inflammation and cell proliferation. To elucidate the mechanism underlying Nox1-redox signalling, it is essential to identify cellular targets for Nox1-derived ROS. In the present study, we have demonstrated that Nox1 oxidizes ERp72 and regulates its redox state, rendering ERp72 a preferential downstream target for Nox1. This conclusion can be drawn by the following observations. First, increased Nox1-catalysed ROS production decreased 5-IAF (Figure 1A) or BIAM (Figure 3A) labelling of redox-sensitive cysteine residues of ERp72 and this inhibition was reversed by DPI (Figure 7D). Furthermore, Nox1-specific siRNAs also prevented EGF from inhibiting the reductase activity of ERp72 (Figure 7E). The results suggest that Nox1 plays a critical mediating role in EGF-induced inhibition of ERp72 reductase activity and further supports our observation that Nox1 functionally interacts with ERp72 (Figures 3–6).
Nox1-generated ROS (Figure 3B). Taken together, these results indicate that reactive cysteine residues of ERp72 are susceptible to oxidation by Nox1. Secondly, Nox1 suppressed the insulin reduction activity of ERp72 (Figure 7A), suggesting that Nox1 inactivates the reductase activity of ERp72 through oxidation. This is supported by the fact that EGF-induced inhibition of ERp72 reductase activity was mediated by ligand-stimulated Nox1 (Figure 7D). Finally, Nox1 was associated with ERp72, as determined by the GST-ERp72 pulldown assay, interaction domain studies, co-immunoprecipitation and subcellular co-localization.

Protein interaction studies demonstrated that the N-terminal (residues 1–105) of ERp72 encompassing the putative Ca\textsuperscript{2+}-binding region and a\textsuperscript{a} region is required for its interaction with Nox1 (Figure 5). Of note, PDI has been found to bind to ubiquilin, which possesses a ubiquitin-like domain, through a similar Ca\textsuperscript{2+}-binding site at its C-terminus [25] and one peptide-binding site has been mapped to the PDI sequence corresponding to most of the Ca\textsuperscript{2+}-binding region [26]. Thus these observations, together with those in the present study, suggest that some particular structure essential for the protein interaction exists at the highly acidic Ca\textsuperscript{2+}-binding region. Three conserved TRX motifs (WCGHCK) in ERp72 are thought to catalyse its enzymatic activity [10]. Utilizing site-directed mutagenesis of cysteine residues, we identified that these three motifs similarly contribute to the insulin reduction activity of ERp72 (results not shown), which is consistent with the previous observations [12]. Since the Nox1-binding site is located in the vicinity of the a\textsuperscript{a} region, Nox1 may target a TRX motif in the a\textsuperscript{a} domain under an in vivo situation.

The results of the present study indicate that Nox1 changes ERp72 to an oxidized state through oxidation of reactive cysteine residues, affecting its enzymatic activity. We therefore speculate that Nox1 may be involved in mediating EGF-induced cell signalling by oxidizing ERp72, changing its conformation, and thus altering its affinity for a specific substrate. Such ROS-mediated regulation of redox proteins have been reported previously [8]. For example, formation of disulfide bonds in TRX through ROS-induced oxidation removes TRX from TRX–ASK1 (apoptosis signal-regulating kinase 1) complexes, leading to activation of ASK1 and the subsequent ASK1-dependent apoptosis [27]. Alternatively, changes in the ERp72 reductase activity as a consequence of oxidation by Nox1 could impede its role in protein folding. Indeed, the reductase activity of ERp57, another PDI family protein, is known to regulate the unfolding of major histocompatibility complex class I molecules [20]. Although it is not clear at present which EGF signalling pathway involves the Nox1–ERp72 interaction, it would be of particular interest to explore the biological consequence of EGF-induced inhibition of ERp72 reductase.

Although most of ERp72 resides in the ER, part of ERp72 was detected at the plasma membrane and co-localized with a transmembrane protein Nox1 (Figure 6). Previous studies indicated that PDI family enzymes are not restricted to ER localization despite the presence of ER retention motifs at their C-terminal ends, and they have activities which may differ from those displayed in the ER lumen [8]. PDI has been found on the surface of platelets [28] and ERp72 has also been detected in membrane fractions of neutrophils primed with formyl-methionyl-leucyl phenylalanine [29]. These observations, together with the present findings, imply wider functional roles for PDI enzymes at non-ER compartments. ERp72, like PDI, is known to be secreted when overexpressed in CHO (Chinese-hamster ovary) cells [30]. By immunoblotting, we examined the level of ERp72 proteins in the conditioned medium of Cos-1 and Caco-2 cells, but failed to detect ERp72 even after a 60-fold concentration (results not shown). This makes it unlikely that the smaller fraction of ERp72 detectable at the plasma membrane (Figure 6A) is extracellular ERp72 that has leaked through the secretory pathway. Yet, the redox activities of PDI family proteins have been implicated in multicaloric processes including molecular chaperones, folding of newly synthesized proteins in the ER, the redox state of cell membrane molecules and the secretory pathway [8]. Further studies are needed to clarify what cellular activity ERp72 targets in response to Nox1 redox-signalling.

The findings in the present study provide a new perspective that Nox1 regulates a yet unidentified function of ERp72 through oxidation. Interestingly, a previous report describes that PDI seems to closely associate with NADPH oxidase and regulate its enzymatic activity in vascular smooth muscle cells [31]. Moreover, a TRX-related protein, EFP1 (ER-hand binding protein 1) was identified to be complexed with Duoxs, although the physiological meaning of the association has not yet been clarified [32]. Thus there might be some commonality in that Nox-family-induced cell signalling involves redox-mediated regulatory mechanisms.

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SUPPLEMENTARY ONLINE DATA
A possible biochemical link between NADPH oxidase (Nox) 1 redox-signalling and ERp72

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Figure S1  For legend, see next page

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Figure S1  The retention time of 2-OH-E• standard and the calibration of its amount

2-OH-E• was produced by treatment of HE with superoxide generated by xanthine/xanthine oxidase. First, to determine the amount of xanthine oxidase required for complete oxidation of 50 μM HE, increasing amounts of xanthine oxidase were added to 400 μl of the reaction mixture [100 mM phosphate buffer (pH 7.4), 50 μM HE, 100 μM DTPA and 1 mM xanthine] at 37 °C for 30 min [1]. Samples were then subjected to HPLC analysis as described in the Materials and methods section of the main text. HPLC chromatograms of 2-OH-E• and E• are shown with retention times (A). Possibly, E• was present as a minor impurity in commercial HE [2]. (B), in which HPLC peak intensity of generated 2-OH-E• is shown as a function of xanthine oxidase, indicates that 0.05 units/ml xanthine oxidase was sufficient to convert HE into 2-OH-E• completely. Therefore we assume that the amount of generated 2-OH-E• is equal to that of used HE when less than 50 μM HE is reacted with 0.05 units/ml xanthine oxidase. Next, the varying amounts of HE (0–50 μM) was oxidized by the addition of xanthine oxidase (0.05 units/ml) and the reaction products were analysed by HPLC (C). Finally, by using the data shown in (C), the HPLC peak intensity of 2-OH-E• was calibrated as a function of concentration (D).

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