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Thioredoxin and thioredoxin reductase influence estrogen receptor α-mediated gene expression in human breast cancer cells

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Abstract

Accumulation of reactive oxygen species (ROS) in cells damages resident proteins, lipids, and DNA. In order to overcome the oxidative stress that occurs with ROS accumulation, cells must balance free radical production with an increase in the level of antioxidant enzymes that convert free radicals to less harmful species. We identified two antioxidant enzymes, thioredoxin (Trx) and Trx reductase (TrxR), in a complex associated with the DNA-bound estrogen receptor α (ERα). Western analysis and immunocytochemistry were used to demonstrate that Trx and TrxR are expressed in the cytoplasm and in the nuclei of MCF-7 human breast cancer cells. More importantly, endogenously expressed ERα, Trx, and TrxR interact and ERα and TrxR associate with the native, estrogen-responsive pS2 and progesterone receptor genes in MCF-7 cells. RNA interference assays demonstrated that Trx and TrxR differentially influence estrogen-responsive gene expression and that together, 17β-estradiol, Trx, and TrxR alter hydrogen peroxide (H_2O_2) levels in MCF-7 cells. Our findings suggest that Trx and TrxR are multifunctional proteins that, in addition to modulating H_2O_2 levels and transcription factor activity, aid ERα in regulating the expression of estrogen-responsive genes in target cells.

Introduction

Eukaryotic cells consume oxygen and produce reactive oxygen species (ROS) as by-products of normal cellular metabolism (Powell *et al*. 2005). ROS include a number of chemically reactive oxygen derivatives including superoxide and hydrogen peroxide (H_2O_2), which are less reactive, and hydroxyl radical, which is highly reactive. The initial product of oxygen metabolism, superoxide, is dismutated to H_2O_2 in cells by superoxide dismutase (SOD). The H_2O_2 is then converted to H_2O and O_2 by catalase, glutathione peroxidase, and peroxiredoxins, which include thioredoxin (Trx) peroxidases (Beckman *et al*. 1990, Webster *et al*. 2001, Yoshida *et al*. 2003, Smart *et al*. 2004, Hashemy *et al*. 2006).

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Declaration of interest

None of the authors has a conflict of interest.

ROS are needed to serve as molecular messengers in cell-signaling pathways and in the immune system to target pathogens (Lehnert & Iyer 2002, Feinendegen 2005, Goldstein *et al*. 2005). At low concentrations, superoxide and H_2O_2 are effective stimulators of cell growth (Burdon 1995). However, if H_2O_2 is not effectively eliminated, hydroxyl radicals can accumulate and damage proteins, lipids, and DNA (Halliwell & Gutteridge 1985, Storz *et al*. 1990). Increased ROS accumulation has also been linked to tumorigenesis and age-related diseases (Kirkwood & Austad 2000, Toussaint *et al*. 2002) as well as decreased cell survival (Salganik 2001). Thus, oxygen radicals have beneficial as well as detrimental effects.

In order to avoid ROS accumulation and its damaging effects, cells express a battery of oxidative stress response proteins that dissipate oxygen radicals (Halliwell & Gutteridge 1985, Storz *et al*. 1990). Trx is an oxidative stress response protein that activates i) transcription factors in order to alter gene expression and ii) peroxiredoxins, so that cellular H_2O_2 can be diminished (Webster *et al*. 2001, Arner & Holmgren 2006). Like the proteins that it reduces, Trx itself must be reduced in order to activate other proteins. Trx reductase (TrxR) utilizes NADPH to reduce and activate Trx as well as other proteins (Mustacich & Powis 2000). By maintaining protein thiols in the reduced state, Trx and TrxR help to maintain a reduced cellular environment and active transcription factors (Holmgren 1979, 1985, Das *et al*. 1997).

Zinc finger proteins are particularly susceptible to oxidation. Oxidation of zinc finger proteins diminishes the ability of these proteins to interact with their target DNA sequences and ultimately alters gene expression. Oxidation of two nuclear receptor superfamily members, the glucocorticoid receptor and estrogen receptor α (ERα), diminishes the ability of these proteins to bind to DNA (Makino *et al*. 1996, Hayashi *et al*. 1997).

ERα binds to hormone, dimerizes, interacts directly with its recognition sequences in DNA, estrogen-response elements (EREs), and recruits coregulatory proteins that influence estrogen-responsive gene expression. Because we had previously shown that three other oxidative stress proteins, SOD1, protein disulfide isomerase (PDI), and apurinic/apyrimidinic endonuclease 1 or redox factor-1 (Ape1/Ref-1) influence ERα-mediated gene expression (Schultz-Norton *et al*. 2006, Rao *et al*. 2008, Curtis *et al*. 2009), we were intrigued by the identification of Trx and TrxR in a complex of proteins associated with the DNA-bound ERα (Schultz-Norton *et al*. 2008, 2009) and were interested in determining whether Trx and TrxR might also influence ERα-mediated gene expression. We now show that Trx and TrxR alter estrogen-responsive gene expression and that together, 17β-estradiol (E_2), Trx, and TrxR modulate H_2O_2 levels in MCF-7 human breast cancer cells.

Materials and methods

Cell culture

MCF-7 human breast cancer cells, which express ERα, were maintained in phenol red-containing minimum essential medium (MEM, Invitrogen) with 1× non-essential amino acids (NEAA, Invitrogen), 20 mM HEPES, and antibiotics (penicillin–streptomycin and gentamicin) with 5% calf serum. Cells were switched to phenol red-free MEM with 5% charcoal/dextrantreated calf serum (CDCS, Eckert & Katzenellenbogen 1982), NEAA, and antibiotics for 1–3 days before experiments were initiated. MDA-MB-231 human breast cancer cells, which do not express ERα, were maintained in Leibovitz's L-15 medium (Invitrogen) with the same additives as used for MCF-7 cells. U2 osteosarcoma (U2OS) cells were maintained in MEM with 15% heatinactivated FCS and with the same additives as used for MCF-7 cells. HeLa cervical cancer cells were maintained in DMEM /Nutrient Mixture F-12 Ham with 5% heatinactivated FCS and penicillin–streptomycin.

Isolation and identification of Trx and TrxR

Trx and TrxR were isolated as proteins associated with the ERE-bound ERα using agarose gel shift assays and identified using mass spectrometry analysis as previously described (Schultz-Norton *et al*. 2008, 2009). Three peptides that contained amino acid sequences unique to Trx1 (TAFQEALDAAGDKLVVVDF-SATWCGPCK, PFFHSLSEK, and EKLEATINELV) and 11 peptides that contained amino acid sequence identical to TrxR1 (VMVLDFVTPTPLGTRWGLGGTCVNVGCIPKKLMHQAALLGQALQDSR, MIEAVQNHIGSLNWGYR, KVVYENAYGQFIGPHR, FLIATGERPR, IGEHMEEHGIK, QFVPIKVEQIEAGTPGR, VVAQSTNSEEIIEGEYNTVMLAIGR, IPVTDEEQTNVPYIYAIGDILEDKVELTPVAIQAGR, FGEENIEVYHSYFWPLEWTIPSR, VVGFHVLGPNAGEVTQGFAAALK, and QLDSTIGIHPVCAEVFTTLSVTK) were identified. Together, these peptides account for 45.7 and 49.7% of the total Trx and TrxR amino acid sequences respectively.

Western-blot experiments

Nuclear extracts from human breast (MCF-7 and MDA-MB-231), bone (U2OS), and cervical (HeLa) cancer cells were prepared as described (Wood *et al*. 2001). Ten micrograms of nuclear extract were fractionated on 10–18% SDS-polyacrylamide gels, and transferred to a nitrocellulose membrane, which was probed with a Trx-, TrxR-, ERα- (sc-20146, sc-28321, sc-8002 respectively, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), PR-A- and PR-B- (RM-9102-S1, Lab Vision, Fremont, CA, USA), or GAPDH- (TAB1001, Open Biosystems, Huntsville, AL, USA) specific antibody and a HRP-conjugated secondary antibody. Proteins were detected by the SuperSignal West Femto Maximum Sensitivity Substrate chemiluminescent system (Pierce, Rockford, IL, USA).

Immunocytochemistry

MCF-7 cells were plated onto coverslips in six-well plates containing phenol red-free MEM with NEAA, antibiotics, and 5% CDCS. Three wells were treated with ethanol vehicle or with 10 nM E_2. After 24 h, cells were washed with PBS, fixed in PBS with 4% formaldehyde for 10 min, washed with PBS, permeabilized with PBS containing 0.1% Triton X-100 for 20 min, and washed with PBS containing 0.1% Tween 20 (PBST). Samples were blocked with PBST containing 2% BSA and 2% fetal bovine serum for 30 min, incubated with a Trx- or TrxR-specific antibody (sc-20146 and sc-28321 respectively, Santa Cruz Biotechnologies) for 1 h in a humidified chamber, washed with PBST, incubated with donkey anti-rabbit biotin-SP-conjugated antibody (Trx, 711-066-152) or donkey anti-mouse biotin-SP-conjugated antibody (TrxR, 715-066-150, Jackson Immuno-Research, West Grove, PA, USA) for 30 min, washed with PBST, incubated with DyLight 549-conjugated Streptavidin (016-500-084, Jackson ImmunoResearch) for 30 min in the dark, and washed with PBST. Primary antibodies were omitted in control slides and run in parallel to demonstrate the specificity of the Trx and TrxR antibodies. Samples were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and visualized with a 40X objective using a Leica DM4000 B confocal microscope (Leica Microsystems, Inc., Bannockburn, IL, USA) with the Leica TCS SPE system and Application Suite Advanced Fluorescence software. Three fields were examined in three independent experiments so that nine fields were examined for each treatment.

Immunoprecipitation assays

MCF-7 cells were treated with ethanol or 10 nM E_2 for 0.75 h, washed with PBS, harvested in 20 mM Tris pH 7.4, 10 mM EDTA, 100 mM NaCl, 0.5% NP-40, 1 mM Na_3VO_4, 50 mM NaF, and 1× protease inhibitor cocktail (PIC, Sigma), and then pelleted at 20 800 ***g*** at 4 °C for 10 min. The protein concentration of each supernatant was determined using the Bio-Rad

protein assay (Bio-Rad) with BSA as a standard. One microgram of Trx- or TrxR-specific antibody (sc-18215 or sc-31057 respectively, Santa Cruz Biotechnologies) or a control antibody directed against fluorescein (Immunological Resource Center, University of Illinois, Urbana, IL, USA) was incubated with 500 μg of extract overnight at 4 °C with rotation, incubated with 60 μl of a 50% Protein G Sepharose slurry for 1 h (GE Healthcare, Piscataway, NJ, USA), and centrifuged at 960 ***g*** at 4 °C for 2 min. Samples were washed thrice with 20 mM Tris pH 7.4, 10 mM EDTA, 100 mM NaCl, 0.1% NP-40, 1 mM Na_3VO_4, 50 mM NaF, and 1× PIC before fractionation on SDS-polyacrylamide gels and western analysis with an ERα-specific antibody (sc-543, Santa Cruz Biotechnologies).

Chromatin immunoprecipitation assays

MCF-7 cells were treated with ethanol or 10 nM E_2 for 0.75 or 24 h and incubated with 1% formaldehyde. Cells were processed essentially as described (Curtis *et al*. 2007). Antibodies directed against ERα or TrxR (sc-8002 and sc-31057 respectively, Santa Cruz Biotechnologies) were used for chromatin immunoprecipitation (ChIP). Primers specific to the ERE-containing region of the pS2 gene or two regions of the progesterone receptor (PR) gene (JL Boney-Montoya, YS Ziegler, CD Curtis, JA Montoya & AM Nardulli, unpublished observations), located 205 kb (PR205) or 221 kb (PR221) upstream of the PR-B transcription start site, were used for real-time PCR with iQ SYBR Green Supermix (Bio-Rad) and the iCycler PCR thermocycler. Standard curves were generated using 1000, 5000, 10 000, 50 000, and 100 000 copies of each gene for each primer set in each experiment and run in parallel.

RNA interference experiments

MCF-7 cells were transferred to phenol red-free MEM with NEAA and antibiotics with 5% CDCS 1 day prior to plating. Cells were seeded in 12-well plates 24 h before transfection with siLentFect (Bio-Rad) and transferrin (Sigma). We combined 50 pmol small interfering RNA (siRNA) directed against Trx (117158 or 111300), TrxR (111302 or 41855), or control *Renilla* luciferase (4630, Ambion, Austin, TX, USA) with 500 μl phenol red-free medium and incubated this with the cells for 24 h. Cells were then incubated in phenol red-free medium containing ethanol or 10 nM E_2 for 24 h. For protein analysis, cells were harvested with TNE (10 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM EDTA), lysed in lysis buffer (20 mM Tris pH 8, 1 mM EDTA, 200 mM NaCl, and 0.2% NP-40), and subjected to western blot analysis with a Trx-, TrxR- (sc-20146 and sc-28321, Santa Cruz Biotechnologies), PR-A- and PR-B- (RM-9102-S1, Lab Vision), or GAPDH- (TAB1001, Open Biosystems) specific antibody. For RNA analysis, cells were resuspended in TRIzol (Invitrogen) and processed according to the manufacturer's instructions. cDNA was prepared using the Reverse Transcription System (Promega), and real-time PCR was performed using iQ SYBR Green Supermix and the iCycler PCR thermocycler (Bio-Rad) with primers specific to Trx (5′-CTTTCTTTCATTCCCTCT TG-3′ and 5′-GCATTTGACTTCACA CTCTG-3′), TrxR (5′-TGGAATTGGTGCTTGTG-3′ and 5-′TATCTCTTGAC-GGAATCG-3′), pS2, PR, cyclin G2, cyclin D1, Bcl2, and 36B4 (Curtis *et al*. 2007, Creekmore *et al*. 2008). Standard curves were derived using cDNA equivalents of 0.02, 0.2, 2, and 20 ng of RNA and were run in duplicate with each primer set in each experiment.

H_2O_2 quantitation

MCF-7 cells were treated with control, Trx or TrxR siRNA, and ethanol or E_2 as described for siRNA experiments. Cells were harvested and centrifuged at 960 ***g*** for 5 min at 4 °C. The pelleted cells were resuspended in lysis buffer and spun at 20 800 ***g*** for 5 min at 4 °C. The supernatants were transferred to a 96-well plate and Amplex Red (Invitrogen), which interacts with H_2O_2 to produce the red fluorescent oxidation product resorufin, was used to determine the level of endogenous H_2O_2 (Invitrogen). To derive a standard curve, duplicate

samples, which included 250, 500, 750, and 1000 nM of H_2O_2, were run in parallel. All samples were analyzed with a fluorescence plate reader using 544 nm for excitation and 590 nm for fluorescence detection. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad) and used to normalize each sample for any changes in protein levels that might occur in response to E_2.

Statistical analysis

SAS 9.1 Basic Statistics (SAS Institute, Cary, NC, USA) was used for statistical analysis. One-way ANOVA was used to determine whether there were differences between control and experimental groups. A *P*-value ≤0.05 was considered to be statistically significant.

Results

Trx and TrxR are present in cytoplasmic and nuclear compartments

We originally identified Trx and TrxR as proteins associated with the ERE-bound ERα (Schultz-Norton *et al*. 2008, 2009). Although HeLa nuclear extracts had been utilized in these initial experiments, we examined the expression of these two proteins in various cultured cell lines that have been used to study estrogen responsiveness (Druege *et al*. 1986, Green *et al*. 1986, Greene *et al*. 1986, Katzenellenbogen *et al*. 1987). Western blot analysis with Trx- and TrxR-specific antibodies demonstrated that both proteins are expressed in ERα-positive (MCF-7) and ERα-negative (MDA-MB-231) human breast cancer cells, U2OS, and, as expected, HeLa cervical cancer cells (Fig. 1A). Interestingly, when MCF-7 cells were treated with 10 nM E_2 for 24 h, the expression of Trx, but not TrxR, was enhanced (Fig. 1B). A GAPDH-specific antibody was used to demonstrate that similar amounts of protein were loaded in each lane.

ERα resides in the nuclei of MCF-7 cells (Schultz-Norton *et al*. 2006). Although Trx and TrxR have been localized in the nuclei of cultured cells, they have more often been described as cytoplasmic proteins (Arner & Holmgren 2000, Nordberg & Arner 2001, Yoshida *et al*. 2003). To determine whether Trx and TrxR were present in the nuclear compartment of MCF-7 cells where they might be able to interact with ERa and influence gene expression, immunocytochemistry was performed. As seen in Fig. 1C, Trx and TrxR were expressed in the cytoplasm, but were also present in the nuclei of MCF-7 cells. Exposure of MCF-7 cells to E_2 for 24 h dramatically increased Trx expression. The increase in Trx expression might be expected since it has been reported that ERa is associated with the Trx gene in MCF-7 cells (Carroll *et al*. 2005). No changes were observed in TrxR expression or the localization of Trx and TrxR with hormone treatment. Thus, although previous studies have reported the ability of ionizing radiation, nitric oxide, or oxidative stress to induce translocation of Trx to the nucleus (Hirota *et al*. 1999, Wei *et al*. 2000, Arai *et al*. 2006), E_2 did not alter the localization of Trx or TrxR in MCF-7 cells. These findings are consistent with those reported in the Human Protein Atlas (www.proteinatlas.org).

Endogenously expressed Trx, TrxR, and ERα interact

Because Trx and TrxR were originally isolated in a large complex associated with the DNA-bound ERα using HeLa nuclear extracts (Schultz-Norton *et al*. 2008, 2009), we determined whether endogenously expressed Trx, TrxR, and ERα from MCF-7 cells could interact. Trx- and TrxR-specific antibodies were used to immunoprecipitate the proteins from MCF-7 extracts, and then western analysis was performed with an ERα-specific antibody. ERa was immunoprecipitated with Trx- (Fig. 2A, lanes 5 and 6) and TrxR- (Fig. 2B, lanes 5 and 6) specific antibodies. In contrast, a control antibody directed against fluorescein was unable to immunoprecipitate ERα regardless of whether cells had or had not been exposed to E_2 (lanes

3 and 4). These studies demonstrate that endogenously expressed Trx and TrxR associate with ERα in the absence and in the presence of hormone.

TrxR associates with endogenous estrogen-responsive genes

The interaction of endogenously expressed ERα, Trx, and TrxR (Fig. 2) and the association of Trx and TrxR with the DNA-bound ERα *in vitro* (Schultz-Norton *et al*. 2008,2009) led us to investigate whether these proteins associate with native estrogen-responsive genes in MCF-7 cells. Using ChIP assays, we previously demonstrated that ERα and other coregulatory proteins associate with an ERE-containing region of the pS2 gene and with two regions of the PR gene located 205 kb (PR205) and 221 kb (PR221) upstream of the PR-B transcription start site (JL Boney-Montoya, YS Ziegler, CD Curtis, JA Montoya & AM Nardulli, unpublished observations, Schultz-Norton *et al*. 2006,2007,Curtis *et al*. 2007,Creekmore *et al*. 2008,Rao *et al*. 2008). PR205 and PR221 contain one and two imperfect EREs respectively.

Significantly more ERa (Fig. 3A) and TrxR (Fig. 3B) were associated with the ERE-containing regions of the pS2 and PR genes in the presence than in the absence of E_2, suggesting that TrxR may influence estrogen responsiveness of these genes by associating with the DNA-bound ERα in native chromatin. However, we were unable to detect any significant change in the association of Trx with the pS2 and PR genes in the absence and in the presence of E_2 using three different Trx-specific antibodies (data not shown). This could result from the inability of the Trx-specific antibodies to effectively immunoprecipitate Trx (as suggested in Fig. 2A), a transient association of Trx with these gene regions, and/or the relatively small size of Trx (12 kDa), which could make it less accessible to antibody and more susceptible to epitope masking. No changes were observed in the association of ERα or TrxR with the internal control gene, 36B4, which was used for normalization of each sample.

Trx and TrxR influence endogenous estrogen-responsive gene expression

To determine more directly whether Trx and TrxR influence the expression of endogenous, estrogen-responsive genes, RNA interference assays were performed to individually knock down Trx and TrxR expression. MCF-7 cells were transfected with siRNA directed against endogenously expressed Trx or TrxR mRNA. In addition, a control siRNA directed against *Renilla* luciferase, which is not expressed in these cells, was utilized. Trx and TrxR siRNA successfully reduced the protein and mRNA levels of Trx (Fig. 4) and TrxR (Fig. 5) respectively. When control siRNA was used, pS2, PR, cyclin D1, and Bcl2 mRNA and PR protein levels were increased and cyclin G2 mRNA levels were decreased in the presence of E_2 (Figs 4 and 5). These findings are consistent with earlier studies from our laboratory and others (Westley & May 1987,Nardulli *et al*. 1988,Altucci *et al*. 1996,Kim *et al*. 2000,Stossi *et al*. 2006,Curtis *et al*. 2007,Creekmore *et al*. 2008,Rao *et al*. 2008).

While the E_2-induced increase in pS2 mRNA expression was reduced when Trx siRNA was included, PR mRNA and protein levels were further enhanced (Fig. 4). The E_2-induced repression in cyclin G2 was enhanced, resulting in further reduction in cyclin G2 mRNA expression. In contrast, cyclin D1, Bcl2, and ERα mRNA levels were unaltered when Trx expression was reduced. The internal control gene, 36B4, which contains no apparent ERα-binding sites, was unaffected by E_2 or the Trx siRNA (Fig. 4B).

When TrxR was knocked down, pS2 and cyclin D1 mRNA levels decreased and PR and Bcl2 mRNA levels as well as PR protein increased in the presence of hormone (Fig. 5). Cyclin G2 mRNA levels were not significantly altered when TrxR expression was reduced and ERα mRNA levels were decreased in the absence, but not in the presence of E_2. Again,

36B4 mRNA levels were unaffected by E_2 or TrxR siRNA (Fig. 5B). The Trx and TrxR siRNAs were protein specific. Decreasing the level of one protein did not significantly affect the expression of the other (data not shown). Furthermore, two different Trx and TrxR siRNAs produced similar effects on estrogen-responsive gene expression. Taken together, these studies indicate that Trx and TrxR have gene-specific, rather than global effects, on estrogen-responsive gene expression.

Trx and TrxR alter H_2O_2 levels in MCF-7 cells

It is well established that Trx influences ROS distribution by activating antioxidant enzymes that convert H_2O_2 to H_2O (Tam *et al*. 2003, Arner & Holmgren 2006). Since E_2 increases Trx expression (Figs 1 and 4), we hypothesized that E_2 treatment might affect H_2O_2 levels in MCF-7 cells. Furthermore, since TrxR is required to activate Trx, it seemed possible that TrxR might also play a role in regulating H_2O_2 levels. To test these hypotheses, MCF-7 cells that were exposed to control, Trx, or TrxR siRNA in the absence and in the presence of E_2 and H_2O_2 levels were measured. When control siRNA was used, H_2O_2 levels were higher in the absence than in the presence of E_2 (Fig. 6). This decreased level in H_2O_2 after treatment of MCF-7 cells with E_2 for 24 h most likely results from the dissipation of ROS by the E_2-mediated increase in antioxidant proteins (Mobley & Brueggemeier 2004, Rao *et al*. 2008).

When Trx or TrxR expression was knocked down and cells were treated with ethanol, H_2O_2 levels were significantly reduced. However, when cells were treated with E_2 and Trx or TrxR siRNA, H_2O_2 levels were enhanced. These studies demonstrate that individually and collectively Trx, TrxR, and E_2 alter H_2O_2 levels in MCF-7 cells.

Discussion

Previous studies have shown that Trx and TrxR help to dissipate ROS, maintain a reduced intracellular environment, and protect cellular macromolecules from oxidative damage (Holmgren 1985, Holmgren & Bjornstedt 1995, Osborne *et al*. 2001, Lincoln *et al*. 2003, Smart *et al*. 2004). We now demonstrate that endogenously expressed Trx and TrxR interact with ERα and alter estrogen-responsive gene expression and that together, Trx, TrxR, and E_2 influence H_2O_2 levels in MCF-7 human breast cancer cells.

Effect of Trx and TrxR on H_2O_2 levels

Although a previous study reported that ROS levels increase when MCF-7 cells are treated with 100 mM E_2 for 15 min (Felty *et al*. 2005), we, in fact, observed an E_2-dependent reduction in H_2O_2 levels when MCF-7 cells had been exposed to 10 nM E_2 for 24 h. While the 1000-fold difference in E_2 concentrations used in these two studies might account for some of the difference observed, we believe that the diminished H_2O_2 levels observed after 24 h of E_2 treatment are primarily due to the increased expression of oxidative stress proteins such as SOD1 (Rao *et al*. 2008) and Trx (Figs 1 and 4), increases that would not be observed after a 15 min exposure to E_2. In addition, although an earlier study monitored H_2O_2 levels after the addition of exogenous H_2O_2 to the culture media (Mobley & Brueggemeier 2004), these experiments are distinctly different from our studies in which cells were exposed to vehicle or hormone, and endogenous production of H_2O_2 was measured. Overall, our findings support the idea that E_2 plays an important role in regulating H_2O_2 levels in MCF-7 cells by modulating the expression of oxidative stress proteins.

Effects of Trx and TrxR on estrogen-responsive gene expression

The capacity of Trx to reduce peroxiredoxins, which convert H_2O_2 to H_2O (Arner & Holmgren 2006), helps to maintain a reduced intracellular environment and ensure that

transcription factors are in a reduced, active state. Thus, Trx along with its activator, TrxR, help in the overall maintenance of transcription factorbinding activity.

The zinc fingers of ERα provide the specificity required for recognizing and interacting with ERE-containing DNA, but are sensitive to oxidative stress (Webster *et al*. 2001). Although oxidation of ERα by ROS or the oxidizing agent diamide inhibits the ability of the receptor to interact with ERE-containing DNA, its DNA-binding capacity can be restored by the reducing agent dithiothreitol or Trx (Hayashi *et al*. 1997). The ability of Trx and TrxR to help maintain ERα structure and function is evident in the altered estrogen-responsive gene expression when either protein is knocked down (Figs 4 and 5). Trx also plays an active role in maintaining the DNA-binding activity of other transcription factors including NF-κB, cAMP response element binding protein, p53, Sp1, AP-1 proteins, and the glucocorticoid receptor (Matthews *et al*. 1992, Wu *et al*. 1996, Hayashi *et al*. 1997, Hirota *et al*. 1997, Liang *et al*. 1998, Ueno *et al*. 1999, Webster *et al*. 2001).

Although we were unable to detect a difference in the association of Trx with the pS2 and PR genes in the absence and in the presence of hormone, the isolation of Trx in a complex with the DNA-bound ERα (Schultz-Norton *et al*. 2008, 2009), the immunoprecipitation of ERα with a Trx-specific antibody in the absence and in the presence of hormone (Fig. 2A), and the ability of Trx to reduce ERα and enhance its binding to DNA (Hayashi *et al*. 1997) suggest that Trx associates with ERα at target genes. The E_2-induced increase in the association of TrxR with the pS2 and PR genes in the presence of hormone (Fig. 3B) could help to ensure that any Trx associated with these gene regions is active and capable of reducing ERα and its associated coregulatory proteins and influencing transcription. Thus, TrxR, through its modulation of Trx activity, may play a role in reducing proteins and maintaining a reduced environment (Arner & Holmgren 2000, Nordberg & Arner 2001). In addition, TrxR has been referred to as a ‘redox sensor’ (Sun *et al*. 1999) and may serve in this capacity to help modulate estrogen-responsive gene expression.

Because of their interdependent nature, it was somewhat surprising that Trx and TrxR would have different effects on cyclin G2, cyclin D1, and Bcl2 gene expression (Table 1). However, it is important to remember that estrogen-responsive genes are regulated not simply by ERα alone, but by a complex array of transcription factors and coregulatory proteins bound to multiple cis elements in extended gene regions, and that the association of a single transcription factor with a single gene region cannot necessarily be used to predict the transcriptional response.

Biological roles of Trx and TrxR

Given the role of Trx and TrxR in influencing estrogen-responsive gene expression, it is not surprising that these two proteins would influence reproductive function. An earlier study suggested that Trx and TrxR were part of a uterine antioxidant system required for maintaining estrogen responsiveness of the uterus (Deroo *et al*. 2004). These and other studies have shown that E_2 increases uterine expression of Trx and TrxR in rodents and humans (Maruyama *et al*. 1997, 1999, Osborne *et al*. 2001, Deroo *et al*. 2004). In addition to their roles in reproduction, Trx and TrxR have been implicated in cancer prevention (Urig & Becker 2006) and progression (Turunen *et al*. 2004, Biaglow & Miller 2005, Arner & Holmgren 2006, Fujino *et al*. 2006). The ability of Trx and TrxR to alter H_2O_2 levels (Fig. 6) could help to maintain gene expression by regulating the redox state of critical transcription factors. Furthermore, they could be essential in avoiding oxidative stress and limiting the damage to cellular macromolecules, which has been associated with aging and age-related disease (Harman 1956, 2001, Finkel & Holbrook 2000). In fact, the role of Trx in regulating oxidative stress and influencing the aging process has been previously reported (reviewed in Yoshida *et al*. (2003)). The overall biological importance of these two proteins

is evident in the early embryonic lethality of Trx- and TrxR-null mice (Matsui *et al*. 1996, Jakupoglu *et al*. 2005).

Effect of oxidative stress proteins on estrogen responsiveness

Trx and TrxR cooperate with other enzymes to dissipate intracellular ROS and maintain the capacity of transcription factors to bind to DNA (Fig. 7A, reviewed in Webster *et al*. (2001)). The conversion of superoxide to H_2O_2 by SOD1 is the first line of defense against ROS. TrxR reduces Trx, which in turn activates peroxiredoxins to aid in the conversion of H_2O_2 to H_2O and O_2, and enhances binding of transcription factors including ERα, AP-1 proteins, NFκB, and p53 to their cognate recognition sequences (Xanthoudakis & Curran 1992,Hayashi *et al*. 1997,Jayaraman *et al*. 1997,Webster *et al*. 2001,Nishi *et al*. 2002). PDI catalyzes disulfide bond formation, reduction, and isomerization (Turano *et al*. 2002), and functions as a molecular chaperone for numerous proteins (Lyles & Gilbert 1991,Noiva *et al*. 1993,Wang & Tsou 1993,Puig *et al*. 1994,Quan *et al*. 1995,Schultz-Norton *et al*. 2006). Ape1 plays a role in DNA repair, redox regulation, and, like Trx, stimulates binding of transcription factors to DNA (Xanthoudakis & Curran 1992,Jayaraman *et al*. 1997,Webster *et al*. 2001,Nishi *et al*. 2002). Each of these proteins, Trx, TrxR, SOD1, PDI, and Ape1, plays a critical role in regulating oxidative stress, and each influences ERα-mediated gene expression in MCF-7 cells (Schultz-Norton *et al*. 2006,Rao *et al*. 2008,Curtis *et al*. 2009). Because of the interdependent nature of these proteins, perturbation in expression of any one protein has the potential to create disequilibrium and alter gene expression and ROS distribution as was observed when Trx or TrxR was knocked down (Figs 4–6).

The current study combined with our previous work (Schultz-Norton *et al*. 2006, 2008, Rao *et al*. 2008, Curtis *et al*. 2009) supports the idea that Trx and TrxR are members of an interconnected network of proteins (Lundstrom & Holmgren 1990, Cheung *et al*. 1999, Wei *et al*. 2000, Webster *et al*. 2001, Atkin *et al*. 2006, Schultz-Norton *et al*. 2006, 2008, Ando *et al*. 2008, Rao *et al*. 2008, Curtis *et al*. 2009), which collectively help to maintain the structural integrity and activity of ERα, its associated coregulatory proteins, and other complex members (Fig. 7B). TrxR is required to maintain Trx in an active, reduced state (Webster *et al*. 2001); Trx reduces Ape1 to bring about changes in transcription factor activity (Wei *et al*. 2000); Ape1 reduces Trx to influence gene expression (Ando *et al*. 2008); together, Trx and TrxR reduce PDI (Lundstrom & Holmgren 1990); PDI prevents misfolding of many proteins including TrxR and SOD1 (Cheung *et al*. 1999, Atkin *et al*. 2006) and acts as a molecular chaperone for ERα (Schultz-Norton *et al*. 2006); and ERα associates with TrxR, PDI, SOD1, and Ape1 at endogenous estrogen-responsive genes (Fig. 3, Rao *et al*. 2008, Schultz-Norton *et al*. 2008, Curtis *et al*. 2009)). Taken together, our studies suggest that ERα serves as a nucleating factor to recruit proteins involved in regulating oxidative stress to estrogen-responsive genes, and that oxidative stress proteins are, in turn, instrumental in altering estrogen-responsive gene expression and redox regulation.

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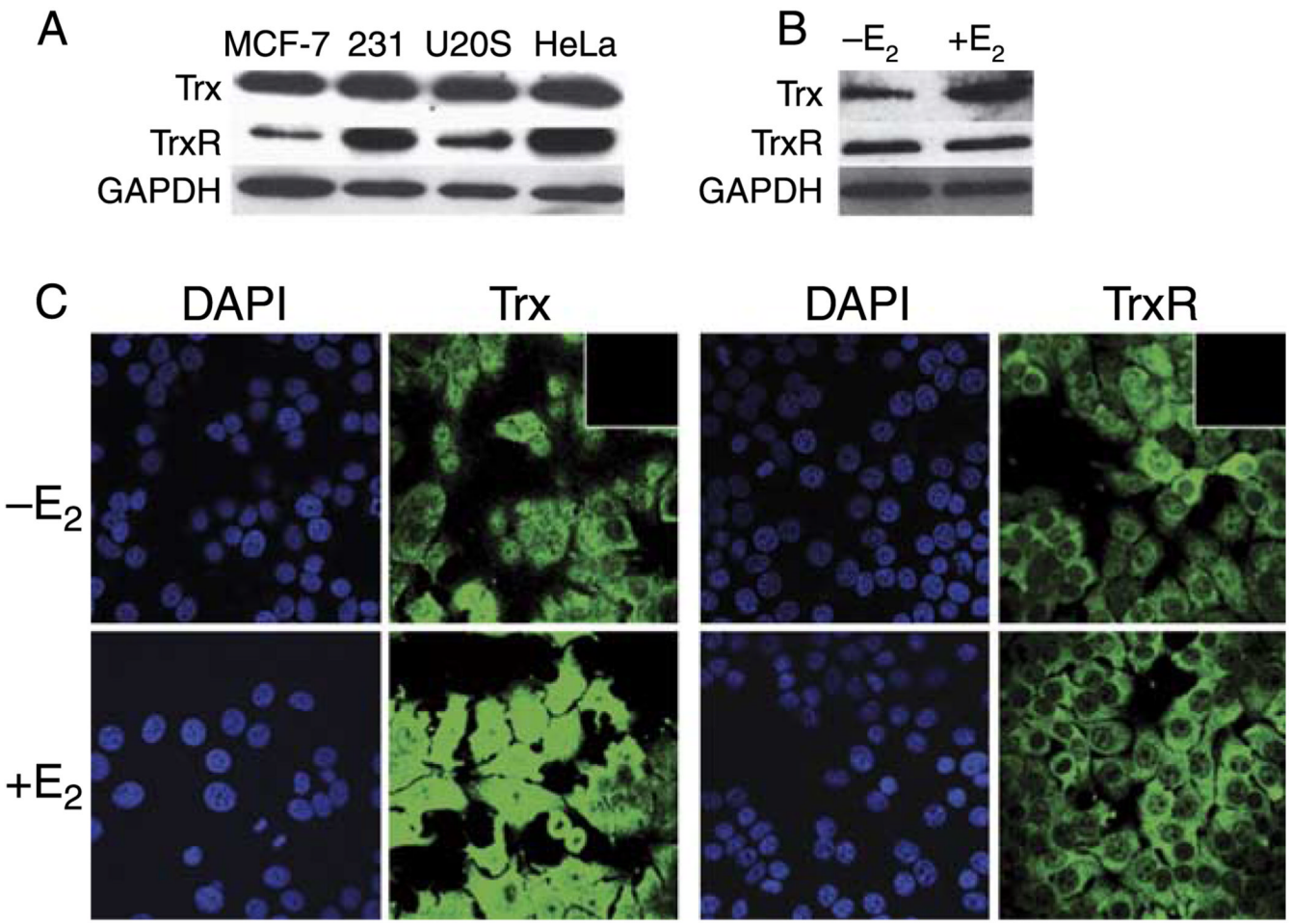

Figure 1.

Trx and TrxR expression and localization. (A) The levels of Trx and TrxR were examined in nuclear extracts (10 μg) from human ERα-positive breast (MCF-7) and ERα-negative breast (MDA-MB-231), U2 osteosarcoma (U2OS), and cervical (HeLa) cancer cells. (B) Trx and TrxR expression was examined in MCF-7 cells that had been treated with ethanol ($-E_2$) or 10 nM E_2 ($+E_2$) for 24 h. Samples were fractionated by SDS-PAGE and subjected to western analysis with a Trx- or TrxR-specific antibody. GAPDH levels were monitored to demonstrate that similar amounts of sample were loaded in each lane. (C) MCF-7 cells were treated with ethanol ($-E_2$) or 10 nM E_2 ($+E_2$) for 24 h. Expression of Trx and TrxR was monitored using immunocyto-chemistry with Trx- and TrxR-specific antibodies. DAPI counter-staining was used to detect cell nuclei. The insert in the upper right hand corner of the $-E_2$ images demonstrated that when the primary antibody was omitted, the secondary antibodies produced no signal. Full colour version of this figure available via http://dx.doi.org/10.1677/JME-09-0053.

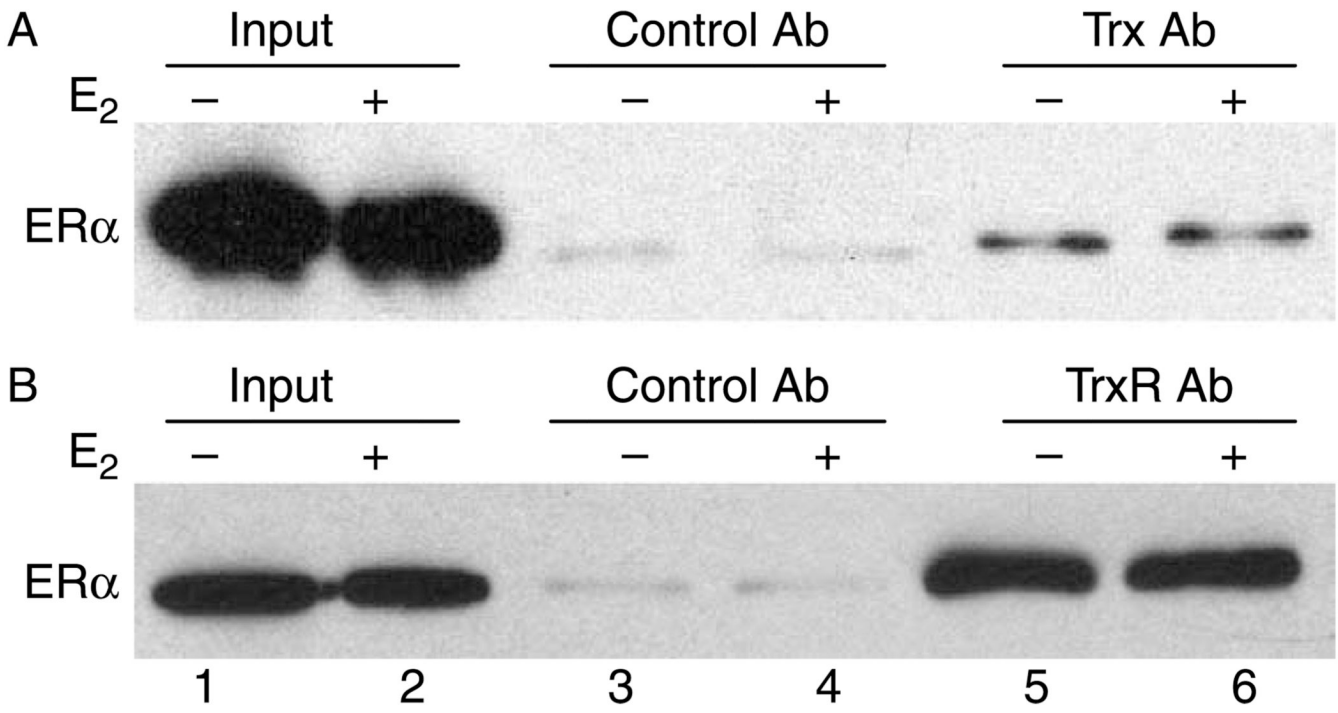

Figure 2.

Endogenously expressed Trx, TrxR, and ERα interact. MCF-7 cells were treated with ethanol ($-E_2$) or 10 nM E_2 ($+E_2$) for 0.75 h and lysed. Cell extracts were incubated with (A) Trx- or (B) TrxR- specific antibody. Specifically bound proteins were eluted and subjected to western analysis with an ERα-specific antibody. MCF-7 extracts (10% input) were included in each experiment for comparison. Data shown are representative of three (A) or six (B) independent experiments.

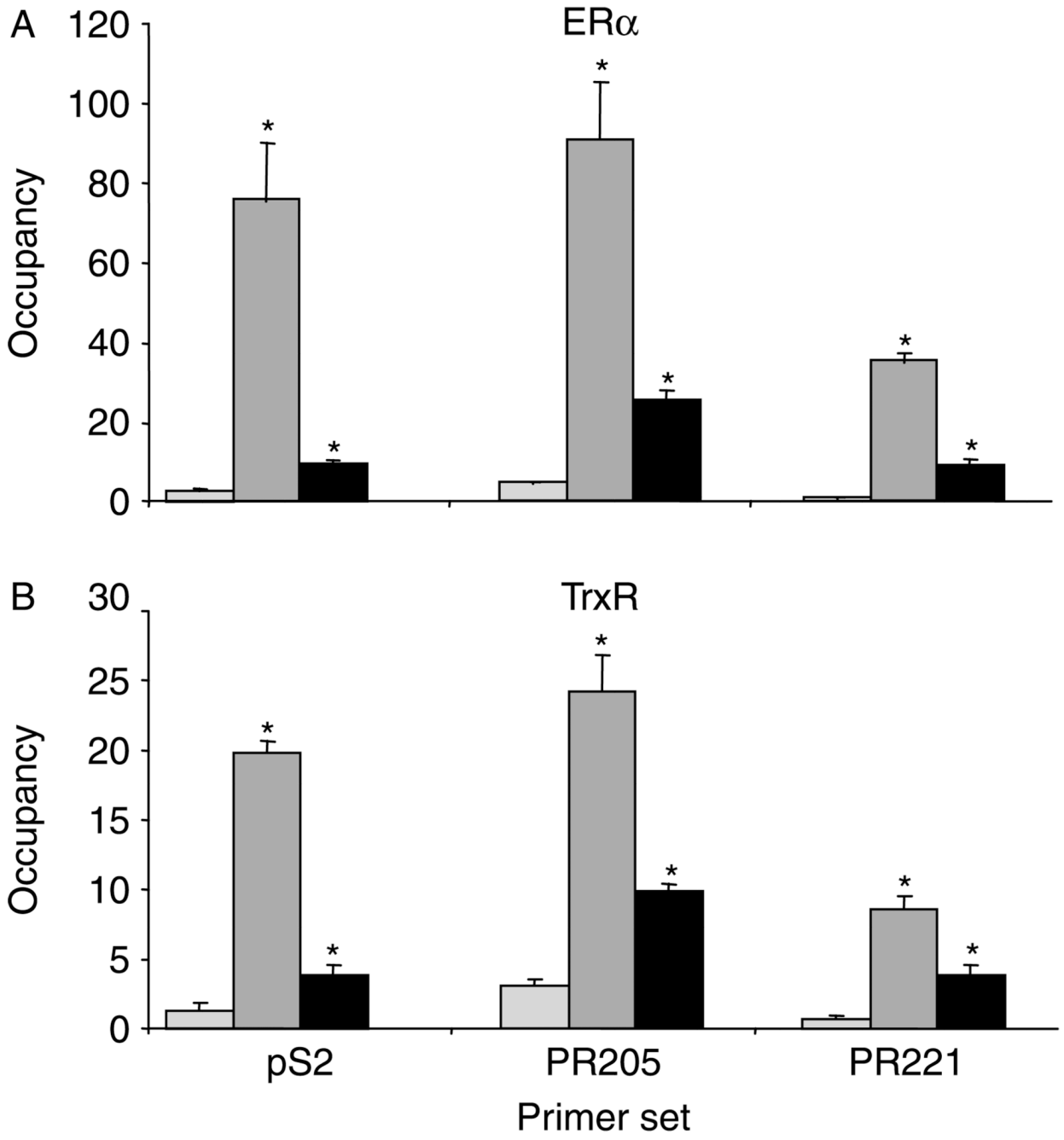

Figure 3.

E_2 increases association of TrxR with endogenous, estrogen-responsive genes. MCF-7 cells were treated with ethanol (light gray bars) or 10 nM E_2 for 0.75 (dark gray bars) or 24 h (black bars) and subjected to ChIP analysis with an (A) ERα-or (B) TrxR-specific antibody. Quantitative real-time PCR was used to examine the association of ERα and TrxR with the ERE-containing regions of the pS2 and PR (PR205 and PR221) genes. Data are presented as the number of copies of each estrogen-responsive gene region pulled down relative to the number of copies of 36B4 gene region pulled down (occupancy). A significant increase in the association of ERα and TrxR with these gene regions in the presence of E_2 is indicated by an. asterisk (*$p \leq 0.05$).

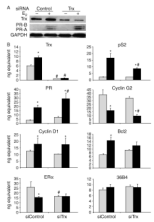

Figure 4.

Knocking down Trx influences estrogen responsiveness. MCF-7 cells were transfected with 50 pmol control or Trx siRNA, treated with ethanol ($-E_2$ and light gray bars) or 10 nM E_2 ($+E_2$ and black bars) for 24 h, and processed for protein or mRNA analysis. (A) Proteins were subjected to western analysis with an antibody that recognizes Trx, PR-A and PR-B, or GAPDH. (B) RNA was isolated and cDNA was synthesized for quantitative RT-PCR analysis with primers specific to Trx, pS2, PR, cyclin G2, cyclin D1, Bcl2, ERα, and 36B4 (internal control) mRNA sequences. Data from three independent experiments, which had been performed in triplicate, were combined and are presented as the mean $\pm_{S.E.M.}$. ANOVA was used to detect significant differences in mRNA levels in response to E_2 ($^{*}P \leq 0.05$) or in response to Trx siRNA ($^{\#}P \leq 0.05$).

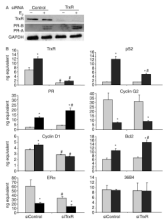

Figure 5.

Knocking down TrxR influences estrogen responsiveness. MCF-7 cells were transfected with 50 pmol control or TrxR siRNA, treated with ethanol (–E_2 and light gray bars) or 10 nM E_2 (+E_2 and black bars) for 24 h, and processed for protein or mRNA analysis. (A) Proteins were subjected to western analysis with an antibody that recognizes TrxR, PR-A and PR-B, or GAPDH. (B) RNA was isolated and cDNA was synthesized for quantitative RT-PCR analysis with primers specific to TrxR, pS2, PR, cyclin G2, cyclin D1, Bcl2, ERα, and 36B4 (internal control) mRNA sequences. Data from three independent experiments, which had been performed in triplicate, were combined and are presented as the mean±$_{S.E.M.}$. ANOVA was used to detect significant differences in mRNA levels in response to E_2 (*$P \leq 0.05$) or in response to TrxR siRNA ($^{\#}P \leq 0.05$).

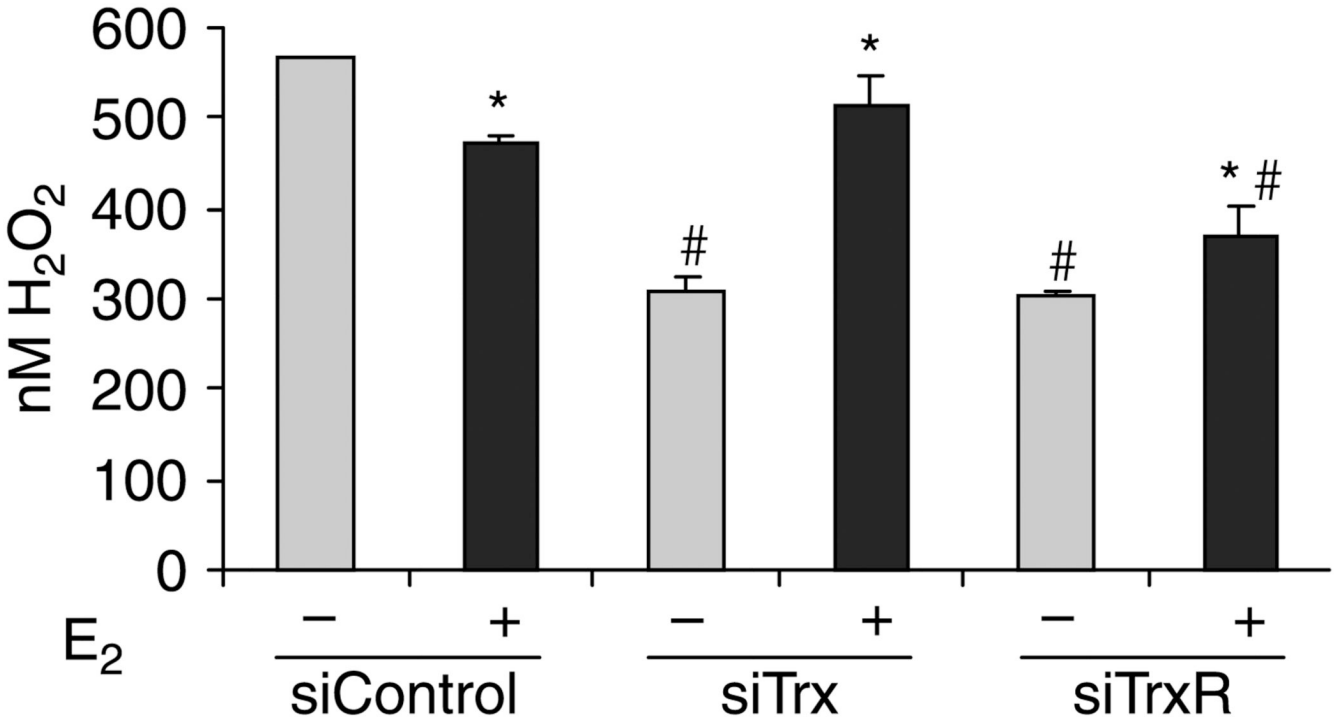

Figure 6.

Trx, TrxR, and E_2 modulate H_2O_2 levels. MCF-7 cells were transfected with 50 pmol control, Trx, or TrxR siRNA and treated with ethanol ($-E_2$) or 10 nM E_2 ($+E_2$) for 24 h. Cell extracts were prepared and incubated with Amplex Red to detect the levels of H_2O_2. Data from three independent experiments were combined and are expressed as the mean $\pm_{S.E.M.}$. ANOVA was used to detect significant differences in the levels of H_2O_2 in the presence of E_2 ($^{*}P \leq 0.05$) or in response to Trx or TrxR siRNA ($^{\#}P \leq 0.05$).

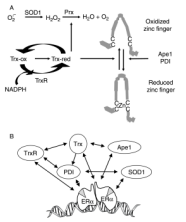

Figure 7.

Oxidative stress response protein forms an interconnected network that alters ROS distribution and influences estrogen responsiveness. (A) Oxidized Trx (Trx-ox) is reduced (Trx-red) by TrxR using NADPH as a cofactor. SOD1 dismutates superoxide to form H_2O_2 and reduced Trx activates peroxiredoxins (Prx) to help eliminate H_2O_2. Trx, Ape1, and PDI reduce zinc finger proteins, enhance interaction with their cognate-binding sites in DNA, and alter transcription. Adapted from Webster *et al*. 2001. (B) Trx, TrxR, SOD1, PDI, and Ape1 form an interconnected network of proteins (Lundstrom & Holmgren 1990, Cheung *et al*. 1999, Wei *et al*. 2000, Webster *et al*. 2001, Atkin *et al*. 2006, Schultz-Norton *et al*. 2006, 2008, Ando *et al*. 2008, Rao *et al*. 2008, Curtis *et al*. 2009) that are recruited to the DNA-bound ERα (Schultz-Norton *et al*. 2008) and influence ERα-mediated gene expression (Schultz-Norton *et al*. 2006, Rao *et al*. 2008, Curtis *et al*. 2009).

Table 1

Regulation of endogenous, estrogen-responsive genes in MCF-7 cells

Gene	Trx	TrxR
pS2	↑	↑
PR	↓	↓
Cyclin G2	↑	–
Cyclin D1	–	↑
Bcl2	–	↓
ERα	–	–
36B4	–	–

RNA interference experiments demonstrate the gene-specific effects of endogenously expressed Trx and TrxR on estrogen responsiveness.