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14. ABSTRACT

We showed that endogenous electrophiles such as the cyclopentenone prostaglandin products of cyclooxygenase-2, and the estrogen metabolite 3,4-estrone quinone (3,4-EQ) are potent irreversible inhibitors of the antioxidant enzyme thioredoxin reductase (TrxR). We present compelling evidence that formation of covalent complexes between electrophiles and TrxR in cells results in the conformational disruption of the tumor suppressor p53. We have prepared an antibody to the TrxR1 covalent complex with 3,4-EQ (anti-3,4-EQ)and have shown it to be a sensitive and selective reagent for the immunochemical detection of proteins in cells treated with the estrogen metabolite. We have also used anti-3,4-EQ in conjunction with anti-rabbit fluorescent secondary antibodies and found that it recognized an antigen in the nucleus of breast cancer cells treated with 3,4-EQ. We believe that anti-3,4-EQ is a promising reagent for detecting electrophilic estrogens that are important biomarkers for breast cancer.

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Introduction

Overexpression of cyclooxygenase-2 (Cox-2) occurs frequently in invasive breast cancers and in adjacent ductal carcinoma *in situ,*[1] and thus may be an early event in carcinogenisis. We have observed that certain products of Cox-2, the electrophilic cyclopentenone prostaglandins (PGs), covalently modify and thereby inhibit the enzyme thioredoxin reductase (TrxR).[2] The antioxidant activity of the thioredoxin-TrxR system is crucial to protecting cells from oxidative stress, which is a major cause of DNA damage and mutagenisis. A highly reactive C-terminal selenocysteine residue makes TrxR uniquely vulnerable to endogenous electrophiles such as PGs.[3] We note the structural similarity between electrophilic PGs and the quinone metabolite of estrogen, E-3,4-Q, and speculate that this compound might also modify TrxR. E-3,4-Q was shown to cause formation of reactive oxygen species (ROS) and single-strand (ss) breaks in DNA of breast cancer cell lines (formation of ss breaks was <u>not</u> estrogen-receptor mediated).[4] In addition, the expression of UDP-glucoronosyl transferase, which facilitates the removal of estrogens from the cell, is dramatically decreased in invasive breast cancers when compared to normal mammary epithelium.[5] We also note that certain covalently modified forms of TrxR, while unable to reduce the natural substrate thioredoxin, continue to utilize NADPH[6] and transfer single electrons to molecular oxygen, resulting in the generation of the potent oxidizing species superoxide. We hypothesized that covalent adducts formed between TrxR and PGs and/or E-3,4-Q behave analogously. Thus TrxR, an essential component of the cell's antioxidant defense mechanism, might not only be disabled by endogenous electrophiles, but also transformed into a catalyst for the production of mutagenic ROS.

Rationale/Purpose: The work described herein addresses two important questions that arise from breast cancer chemoprevention studies and risk assessment. These are, why does the consumption of non-steroidal anti-inflammatory drugs (NSAIDs) lower breast cancer risk, and why does estrogen exposure (no children or birth of a first child after the age of 30) increase a woman's chances of developing breast cancer? We proposed that cyclopentenone PGs (the production of which is inhibited by NSAIDs) and the electrophilic estrogen metabolite E-3,4-Q form covalent adducts with TrxR, inhibit its normal antioxidant functions and transform TrxR into a catalyst for the formation of superoxide, thus leaving breast tissue vulnerable to the deleterious effects of ROS. Since TrxR is a selenoprotein our hypothesis is also consistent with the increased cancer risk associated with selenium deficiency. Our studies will determine whether the proposed TrxR adducts actually occur in breast tumors, and will identify critical biomarkers for the conduct of chemoprevention trials utilizing agents such as NSAIDs, selenium supplements, inducers of Phase 2 detoxification enzymes such as curcumin, and antioxidants.

Body

Results from each task of the two Specific Aims is outlined below:

Specific Aim 1.

Task 1. Prepare Estrone-3,4-quinone

Estrone 3,4-quinone was prepared in good yield using the procedure of Abul-Hajj.[7] Analysis by TLC and UV spectrometry were in good agreement with the literature. The material was stored in DMSO solution (concentration determined by UV spectroscopy using ε= 4500) at -80 degrees in small single use aliquots.

Task 2. Determine potency of inhibition of TrxR by E-3,4-Q and PGs and look for evidence of production of superoxide.

In order to carry out Task 2 we first purified TrxR from rat liver using the protocol of Gromer et al.[8] Then we determined the K_I (Table 1) for inhibition of the TrxR-catalyzed reduction of the disulfide substrate dithiobis-5-5'-dinitrobenzoic acid (DTNB) by endogenous electrophiles listed in Figure 1. We also included in our study 4-HNE, another endogenous electrophile produced by the oxidation of cellular lipids. Reaction rates were determined by monitoring the absorbance at 412 nm of a mixture of TrxR, NADPH, DTNB and electrophile.

Compound	K_i
15-deoxy-Δ-12,14-PGJ_2	0.04 μM
PGA2	8 μM
4-HNE	1 μM
3,4-estronequinone	2 μM

Table 1. K_i values for inhibitors of TrxR.

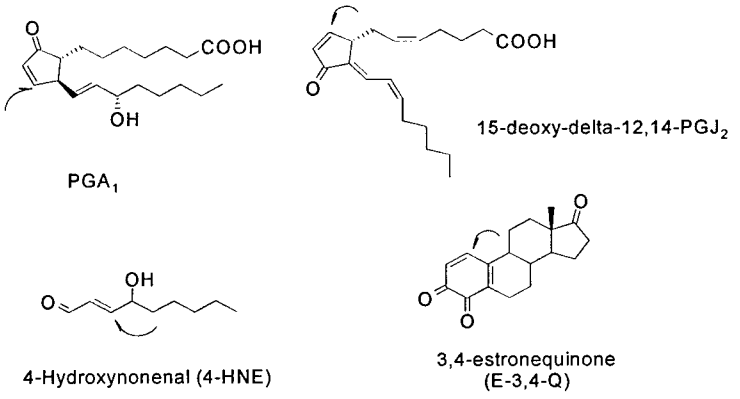


Figure 1. Inhibitors of TrxR. Arrows indicate electrophilic sites.

15-deoxy-Δ-12,14-PGJ_2 (15-d-PGJ_2) was the most potent inhibitor with a K_i of 40 nM. Another cyclopentenone PG, PGA_1, was much less potent. 4-HNE and E-3,4-Q were intermediate in potency with a K_is of 1 μM and 8 μM.

We have data[2] to support the formation of covalent complex between TrxR and endogenous electrophiles. Thus we predicted that incubation of TrxR with NADPH and electrophiles should result in a time-dependent inactivation of the enzyme. This was in fact what we observed (Figure 2). In these experiments, we incubated a solution of TrxR with NADPH and electrophile, then withdrew samples at the indicated times and assayed residual TrxR activity. The enzyme was diluted 50-fold into an assay mixture containing the substrate DTNB at a concentration of $6K_M$. These conditions insured that inhibitor carried-over from the incubation mixture did not interfere with activity measurements. The time-dependent inactivation of TrxR by PGA_2 and 15-d- PGJ_2 fit a model of simple exponential decay with half-time of inactivation of 10 minutes and 12 minutes respectively. The kinetics of inactivation by 4-HNE and E-3,4-Q fit an equation described by two exponential decays, one with a half-time of 20 seconds and the second with an half-time of 7 minutes. In addition, we tested the ability of all compounds to act as substrates of TrxR by monitoring the consumption of NADPH in mixtures of electrophile, TrxR and NADPH. Only E-3,4-Q, showed any activity, and this was extremely modest at 0.08 μmol/min/mg. By comparison reduction of DTNB by TrxR has specific activity of 35 μmol/min/mg.

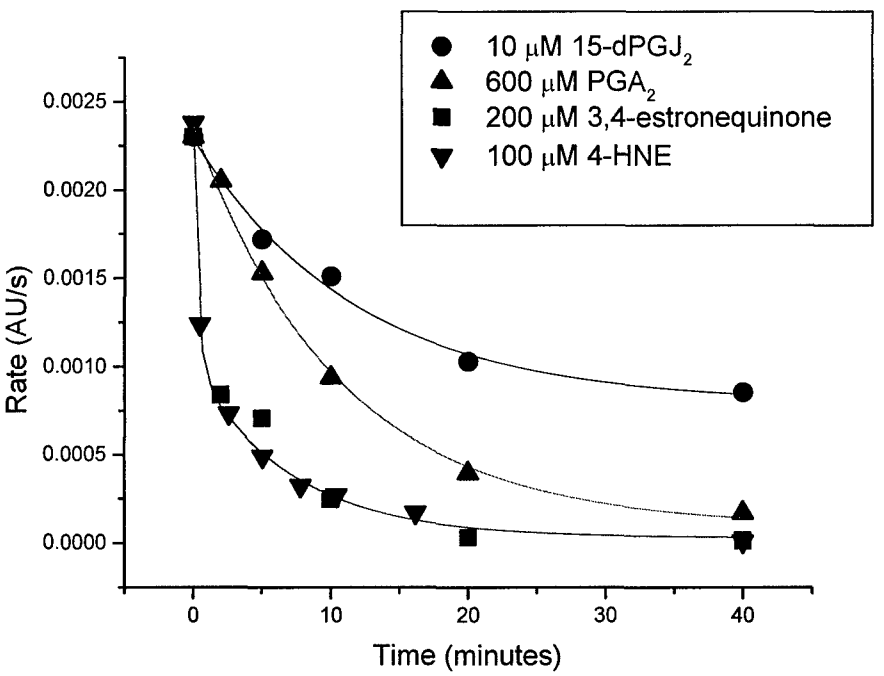


Figure 2. Time-dependent inactivation of TrxR by endogenous electrophiles.

We looked for production of superoxide by TrxR-electrophile complexes using the adenochrome method. Although we were able to reproduce the observations of Nordberg et al. [6] (the dinitrophenyl-TrxR derivative did produce superoxide) we never observed production of superoxide in mixtures of TrxR and any of the endogenous electrophiles that we examined. We then turned to cell culture system developed in our lab for evaluating the consequences of inhibition of TrxR in cells. We showed previously that treatment of colon cancer cells with electrophilic cyclopentenone PGs such as those in Figure 1 caused conformational derangement of the tumor suppressor p53.[9] We later showed that this phenomenon is a result of inhibition of TrxR. For the current project, we examined the same phenomenon in MCF-7 breast cancer cells treated with 15-d-PGJ_2 and E-3,4-Q (Figure 3 panels A and B).

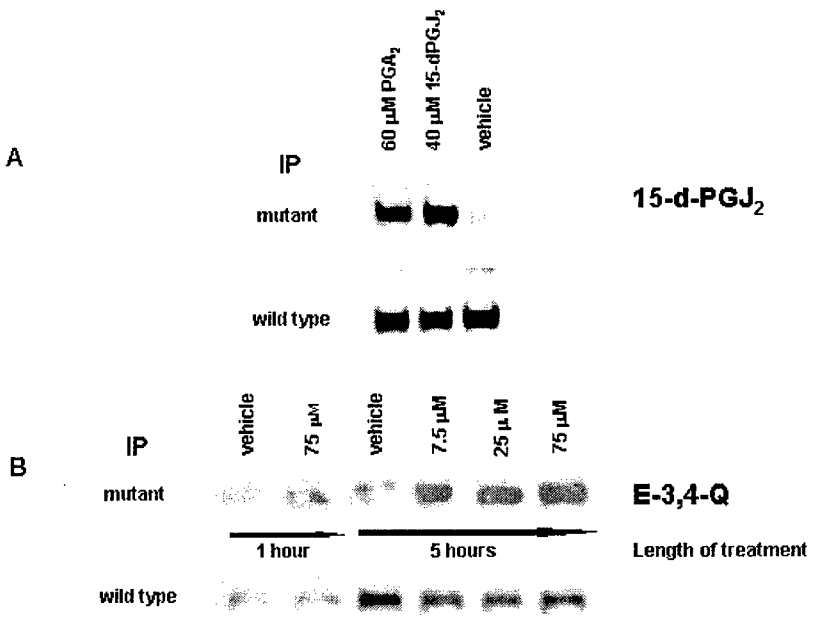


Figure 3. Conformational disruption of p53 in breast cancer cells treated with electrophiles.

This assay takes advantage of two antibodies that selectively immunoprecipitate either the mutant or wild-type conformation of p53. In the experiments shown in Figure 3, cells were treated with the indicated compounds for either 5 hours (in the case of the PGs) or 1 hour and 5 hours with E-3,4-Q. Then the cells were lysed and p53 was immunoprecipitated and analyzed for p53 by western blot. Cells treated with both PGs gave increased the amount of mutant p53 when compared to cells treated with vehicle alone. Cells treated with E-3,4-Q also

showed the same effect on p53 conformation, although the mutant protein appeared much earlier in cells treated with this compound.

We next used this p53 conformational assay to interrogate our hypothesis that the TrxR-electrophile adducts were themselves directly responsible for generation of ROS and consequentially, disruption of p53 conformation. In these new experiments used siRNA directed against TrxR1[1] to support our contention. We generated several siRNAs that target TrxR1 specifically and we found that an siRNA that was effective at attenuating TrxR1 protein levels (Figure 4) and TrxR1 activity (Figure 5) in RKO cells. Then, we generated a scrambled siRNA from the sequence, ensured it would not align with other sequences in the Genbank database (using the small exact match BLAST) and this was designated as the control. This control has no effect on TrxR1 protein expression.

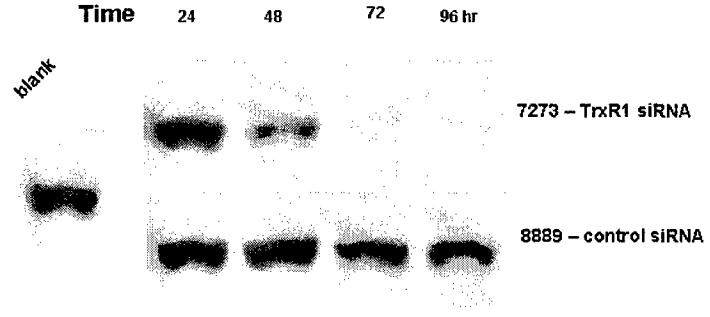


Figure 4. Immunohistochemical analysis of TrxR1 in RKO cells treated with TrxR1 siRNA.

We used these siRNAs in expermiments designed to test the effects of reduced TrxR1 on p53 conformation. We found no difference in the amount of mutant p53 in cells treated with either TrxR1 or scrambled siRNA (Figure 6 panel A). Then, we tested the effect of TrxR1 knock-down on PGA_1-induced p53 conformational change. We believe that the results illustrated in panel B are consistent with our contention that the modified form of TrxR1 mediates the effects on p53 conformation observed in PG-treated cells There was less mutant p53 in cells treated with TrxR1 siRNA and PGA_1 than in cells treated with

[1] There are 3 forms of TrxR in mammalian cells. TrxR1, the cytosolic form is the topic of this report. TrxR2 is the mitochondrial form and TrxR3 is a testis-specific form.

scrambled siRNA and PGA_1. It seems that reduced levels of TrxR1 protein actually makes p53 less susceptible to conformational derangement after PG treatment.

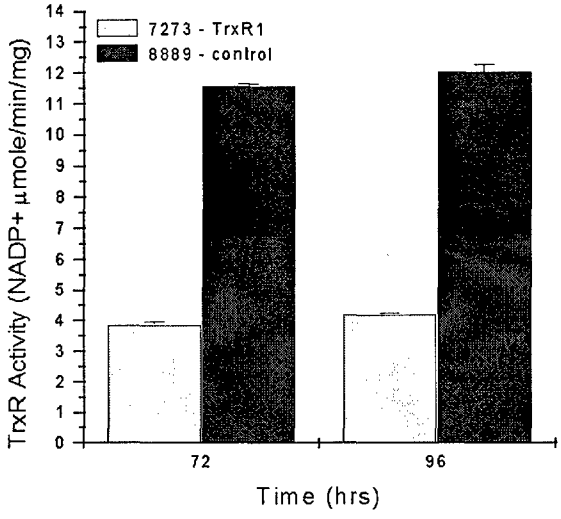


Figure 5. TrxR1 activity in RKO cells transfected with TrxR1 and scrambled siRNA.

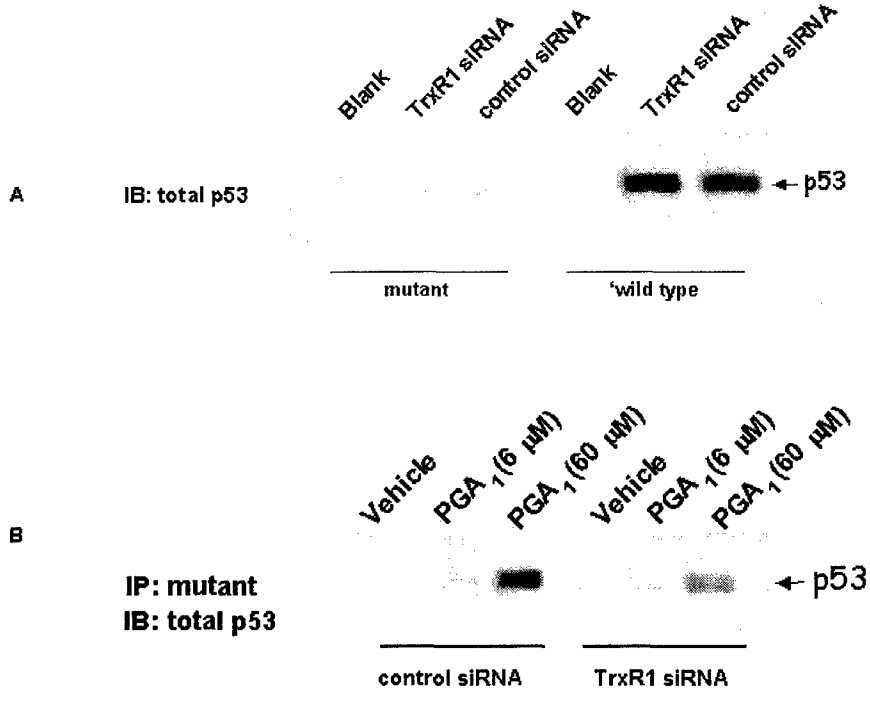


Figure 6. Effects of TrxR1 siRNA on p53 conformation in PG-treated cells.

The p53 conformational assay also allows us to interrogate the system for production of superoxide in cells. In last year's report, we reported the results of an assay in RKO cells pre-treated with PEG-superoxide dismutase (PEG-SOD), a polyethylglycol-derivitized form of the enzyme that is cell permeable.[10] After the standard 5 hour treatment with PG, the cells were lysed and lysates were immunoprecipitated with antibodies for mutant and wild-type p53 conformations. The results, shown in Figure 7, show that PEG-SOD rescues PG-induced conformational derangement of p53. This result however, is extremely variable (we have seen a similar result in only 3 of 7 experiments). The reason for this variability is not known.

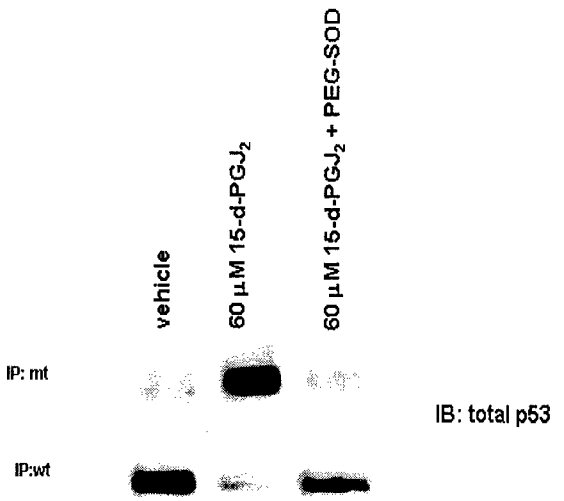
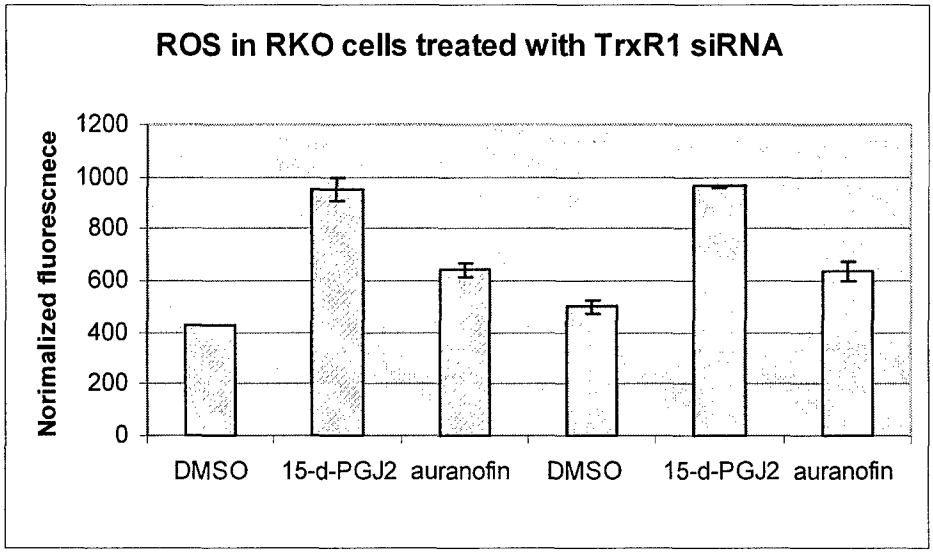


Figure 7. Rescue of PG-induced p53 mutant by SOD.

Another approach to evaluating the role of TrxR1 in superoxide production in cells involves the use of the cell permeable compound dihydrodichlorofluorescene diacetate (DHDCF) which is oxidized to a fluorescent product upon reaction with reactive oxygen species including superoxide. Kondo and co-workers showed that treatment of cells with 15-d-PGJ2 causes increase in the fluorescence of DHDCF.[11] We compared the fluorescence of RKO cells treated with 30 μM 15-d-PGJ2 and depleted of TrxR1 by siRNA treatment with cells treated with 15-d-PGJ2 and control siRNA (Figure 8). We verified the decrease in TrxR levels in cells treated with TrxR siRNA by immunochemical analysis of the same samples. We found no difference in fluorescence in lysates from cells with decreased TrxR1 protein levels compared to control cells. The same result was found when mitochondrial TrxR (TrxR2) was depleted by siRNA

treatment. Thus we conclude that the production of ROS induced by PG treatment is not directly affected by TrxR.



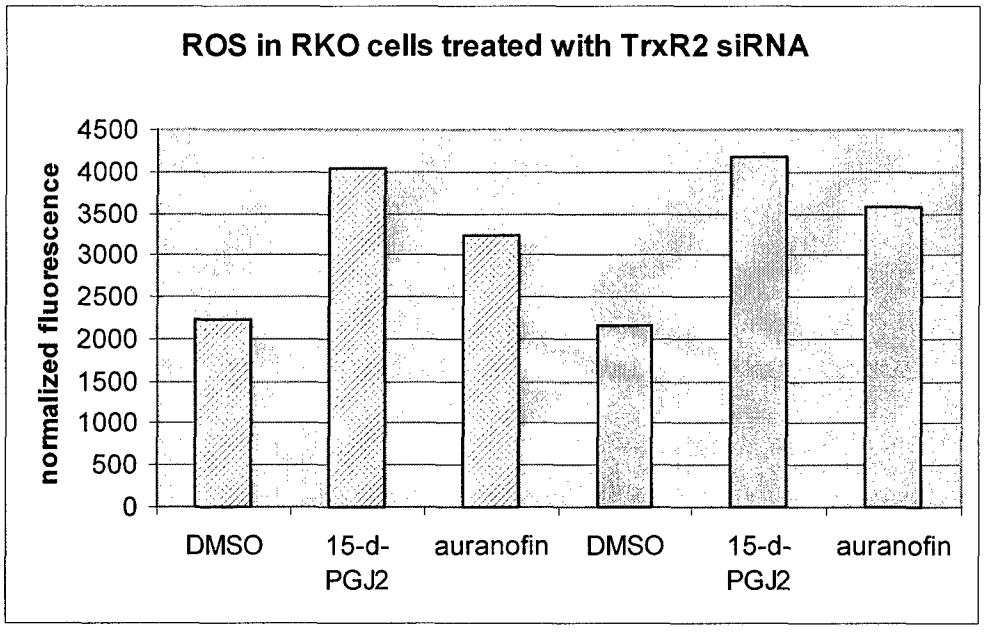


Figure 8. ROS production in cells treated with 15-d-PGJ2 and effects of siRNA knockdown of TrxR1 and TrxR2. Hatched bars represent treatments with TrxR siRNA and solid bars represent treatments with control siRNA.

Specific Aim 2.

Task 1. Prepare covalent adducts to TrxR-derived peptides.

TrxR1 is potently inhibited by auranofin,[8] a thiogold compound which acts by modifying the C-terminal selenocysteine of the enzyme. We used auranofin in experiments with purified TrxR1 to show that PGs also interact with the enzyme at the C-terminus. (Figure 9).

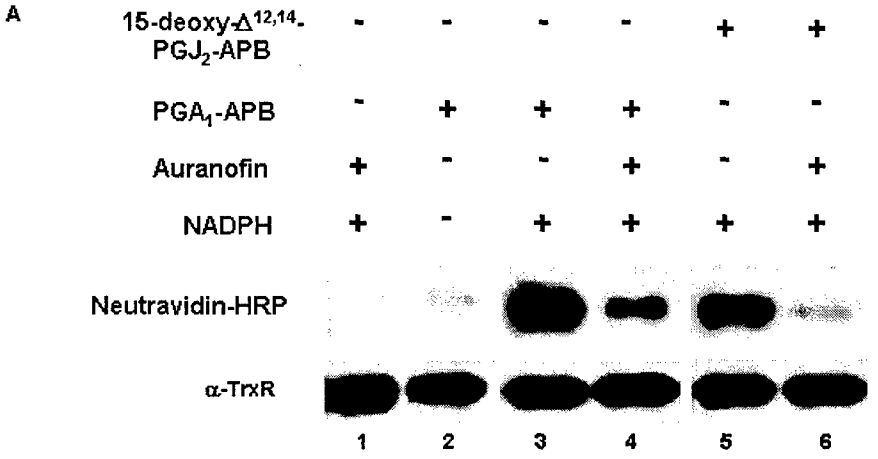


Figure 9. Effects of pre-treatment with auranofin on PG interactions with TrxR1.

In these experiments, purified enzyme was pre-incubated as indicated with or without auranofin, then with PG. The mixture was then analyzed by western blot using neutravidin-HRP to detect covalent modification by the PG and with anti-TrxR1. First we demonstrated a requirement that TrxR1 be reduced with NADPH in order for the biotinylated PG, PGA_1-APB,[2] to be covalently attached (lane 2 vs lane 3). Then, in experiments where NADPH-reduced TrxR1 was first treated with auranofin, followed by either PGA_1-APB or 15-deoxy-Δ-^{12,14}-PGJ_2-APB, we show that inactivation of the C-terminal selenocysteine residue by auranofin blocks the interaction of TrxR with PGs (lane 3 vs lane 4, and lane 5 vs lane 6).

We were fortunate to have a collaborator, Dr. Robert Hondal, prepare the peptide **1** shown in Figure 10 which corresponds to the oxidized form of the C-

terminal 3-amino acids of TrxR1. We used this compound in a number of attempts to prepare adducts of PGs and E-3,4-Q. In all cases, the selenosulfide **1** was first reduced with $NaBH_4$ according to the method of Hondal.[12] We then treated the resulting selenol **2** separately with PGA_2, 15-d-PGJ_2 and E-3,4-Q. In the case of PGA_2, we detected by HPLC only the slow conversion of PGA_2 into PGB_2. This is consistent with the observation that adducts of cyclopentenone PGs to small molecular weight thiols such as cysteine are reversible in solution.[13] The same work showed that higher molecular weight thiols form stable PG adducts due to the reduced molecular motion of the larger adducts. We had hoped that the tripeptide adduct might be large enough to be stable, but unfortunately this did not appear to be the case. PGA_2 also slowly isomerizes in aqueous solution in a competing reaction to the electrophilically hindered and thus unreactive isomer PGB_2. As a consequence, the desired product formed only transiently, if at all, and all of the PG ended up in the thermodynamically most stable form, PGB_2.

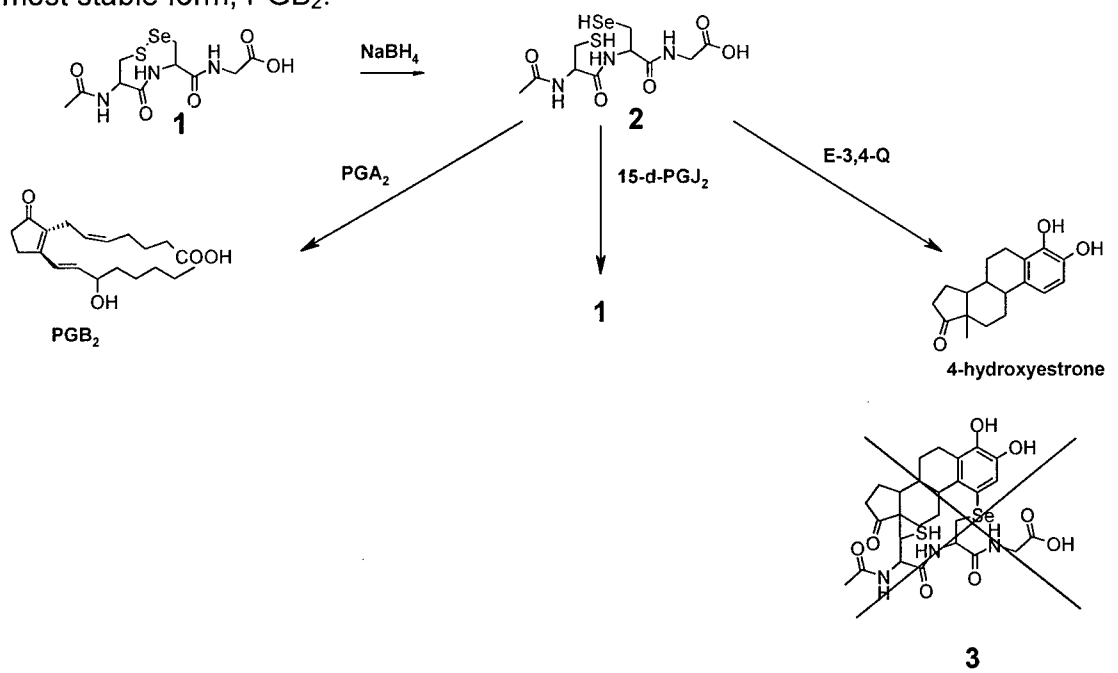


Figure 10. Attempts at preparing TrxR1 peptide adducts.

An analogous reaction with 15-d-PGJ_2 also resulted in no detectable product formation. Analysis of a mixture of selenol **2** and 15-d-PGJ_2 by HPLC showed no detectable formation of a new product. Again we postulate that the desired addition was highly reversible and was probably confounded by the slow re-oxidation of the selenol to the selenosulfide **1.**

Attempts to prepare the adduct **3** between the tripeptide **2** and E-3,4-Q were also unsuccessful. Although we anticipate that **3** should be far more stable than the PG adducts due to the presence of the extremely stable aromatic A-ring of the estrogen adduct, we do not observe anything in the HPLC analysis of the reaction mixture that looks by UV analysis to be compound **3**, we see only loss of E-3,4-Q and formation of a complex mixture. We speculate that E-3,4-Q can be

reduced by the selenol **2** via 1,2-addition to one of the carbonyl carbons followed by attack by the sulfhydryl of the same molecule re-forming the selenosulfide **1** and 4-hydroxy estrone in a reaction directly analogous to that observed (albeit at a very low level) for the TrxR1-catalyzed reaction.

At this stage our supplies of tripeptide **1** were exhausted. To continue the effort to prepare peptide antigens, we decided to change the peptide slightly to include more of the TrxR C-terminal sequence and to make a bioisosteric substitution of sulfur for selenium. The new peptide **4** (Figure11) is much less expensive to prepare in the amounts of material necessary to work out difficulties in chemistry and still have the necessary material required (at least 4 milligrams final product) for antibody preparation. A 150 milligram preparation of this compound was carried out at the University of Utah Peptide Core Facility. We used this material to prepare and purify the 20 mg of bis-dinitrophenyl derivative **5**. This material was analyzed by MALDI mass spectrometric analysis.

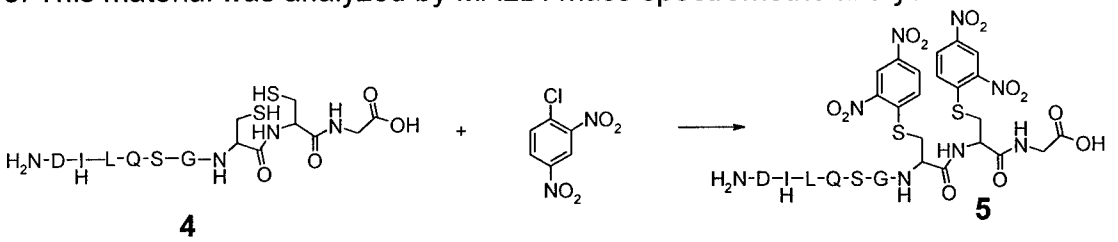


Figure 11. Preparation of bis-dinitrophenyl derivitized peptide **5**.

The preparation of estrone quinone adducts required a change in strategy as well. Stable estrone quinone adducts to cysteine and glutathione are known in the literature.[14] We used the published procedure to prepare the model compound **6**, an N-acetylcysteine derivative (Figure 12). We used a similar procedure to prepare 25 mg of the glutathione ethyl ester derivative **7**.

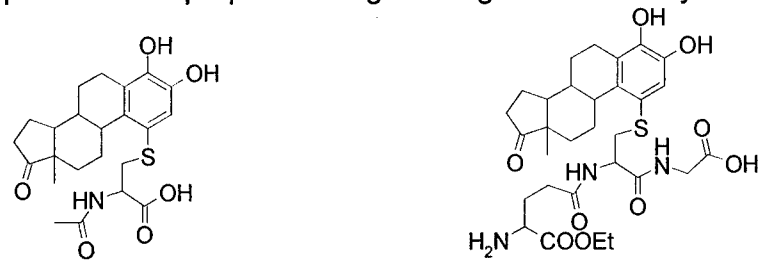


Figure 12. E-3,4-Q adducts.

Task 2. Produce and purify antibodies to TrxR adducts.

Compounds **5** and **7** were sent to the Pocono Rabbit Farm[15] for generation of polyclonal antibodies in rabbits. Each hapten was conjugated via

the amino terminus to keyhole limpet hemocyanin and these conjugates were used for inoculation of the animals. For ELISA analysis of serum, the haptens were conjugated to bovine serum albumin. ELISA analysis of serum from the second bleed is listed in Figure 13. ELISA results from the first bleed ranged from good for the animals inoculated with conjugates of **5** to excellent for one of the animals inoculated with **7**. ELISA results from a second bleed taken one month after the second injection were not substantially different than those shown in Figure 14. A month after a third boost, the animals were sacrificed, exsaguinated and the resulting serum was sent to our labs.

Hapten	Rabbit#	Bleed	Response
5	1	Pre-immune	None
5	1	First immune	24300
5	2	Pre-immune	None
5	2	First immune	24300
7	3	Pre-immune	None
7	3	First immune	72900
7	4	Pre-immune	None
7	4	First immune	218700

Interpretation of Response:
0-1000 poor
1000-10000 fair
10000-25000 promising, possibly a good reagent
25000-50000 excellent
>500000 outstanding

Figure 13. ELISA analysis of serum from animals inoculated against compounds **5** and **7**.

Portions of the serum from each animal were purified on protein A columns.[16] The purified immunoglobulins were tested for utility as reagents for immunochemical analysis by western blot first using purified TrxR1 that was chemically modified with either 3,4-EQ or DNCB. We were somewhat disappointed to find that only immunoglobulins from rabbit number 4 (inoculated with the 3,4-EQ conjugate **7**), referred to in the hereafter as anti-3,4-EQ, gave a band in the lane with 3,4-EQ-modified TrxR1 (Figure14). This antibody was carried on to Task 3.

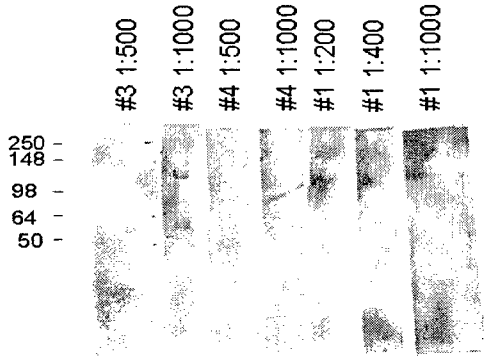


Figure 14. Immunoglobulins from rabbits 1, 3 and 4 tested for utility as reagents for western blot. Rabbit number and dilution of purified immunoglobulin are indicated above the blot.

Task 3. Validate utility of antibodies in breast cancer cell lines treated with PGs and E-3,4-Q.

We first examined the utility of anti-3,4-EQ as a reagent for immunochemical analysis by western blot. MCF7 breast cancer cells were treated with 3,4-EQ at varying concentrations and then the cells were harvested, proteins fractionated by SDS-PAGE and analyzed by western blot using anti-3,4-EQ. We had considerable difficulty finding conditions that resulted in selective reaction with proteins from the 3,4-EQ-treated cells. However, we were eventually successful in demonstrating a selective, dose-dependent interaction of anti-3,4-EQ with proteins from 3,4-EQ-treated cells (Figure 15). Specific bands are detected in the treated cells at 3,4-EQ concentrations below 1 μM. Interestingly, TrxR (MW 55 kD) does not appear to be a major protein detected.

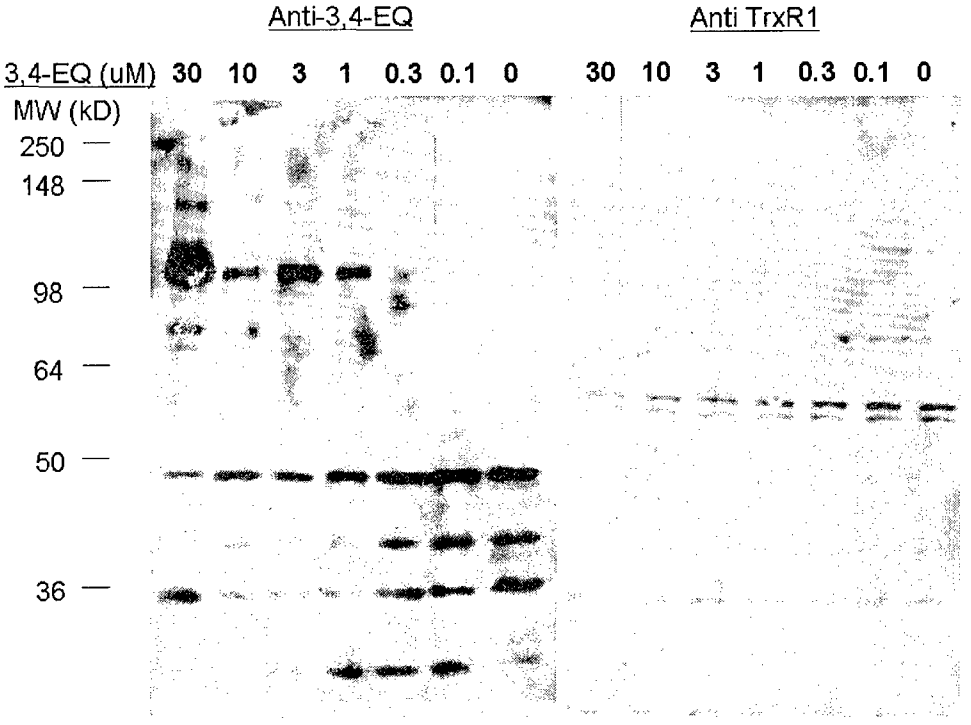


Figure 15. Immunochemical analysis of MCF7 cells treated with the indicated concentrations of 3,4-EQ.

We next tested the utility of anti-3,4-EQ as a reagent for immunocytochemistry. Optimization of conditions for these experiments are still underway, but we did get some indication that the reagent binds with some selectivity to the nuclei of BT20 breast cancer cells treated with 3,4-EQ (Figure 16). The cells treated with 3,4-EQ showed a signal from the Alexa 488-conjugated anti-rabbit secondary antibody in a region that overlapped with the nuclear stain DAPI. The background fluorescence of untreated cells is somewhat high however. Currently we are investigating the inclusion of more stringent washes in our protocol in order to reduce background. We are also considering performing an affinity purification of the serum from rabbit number 3 using a sepharose conjugate of the hapten **7**.

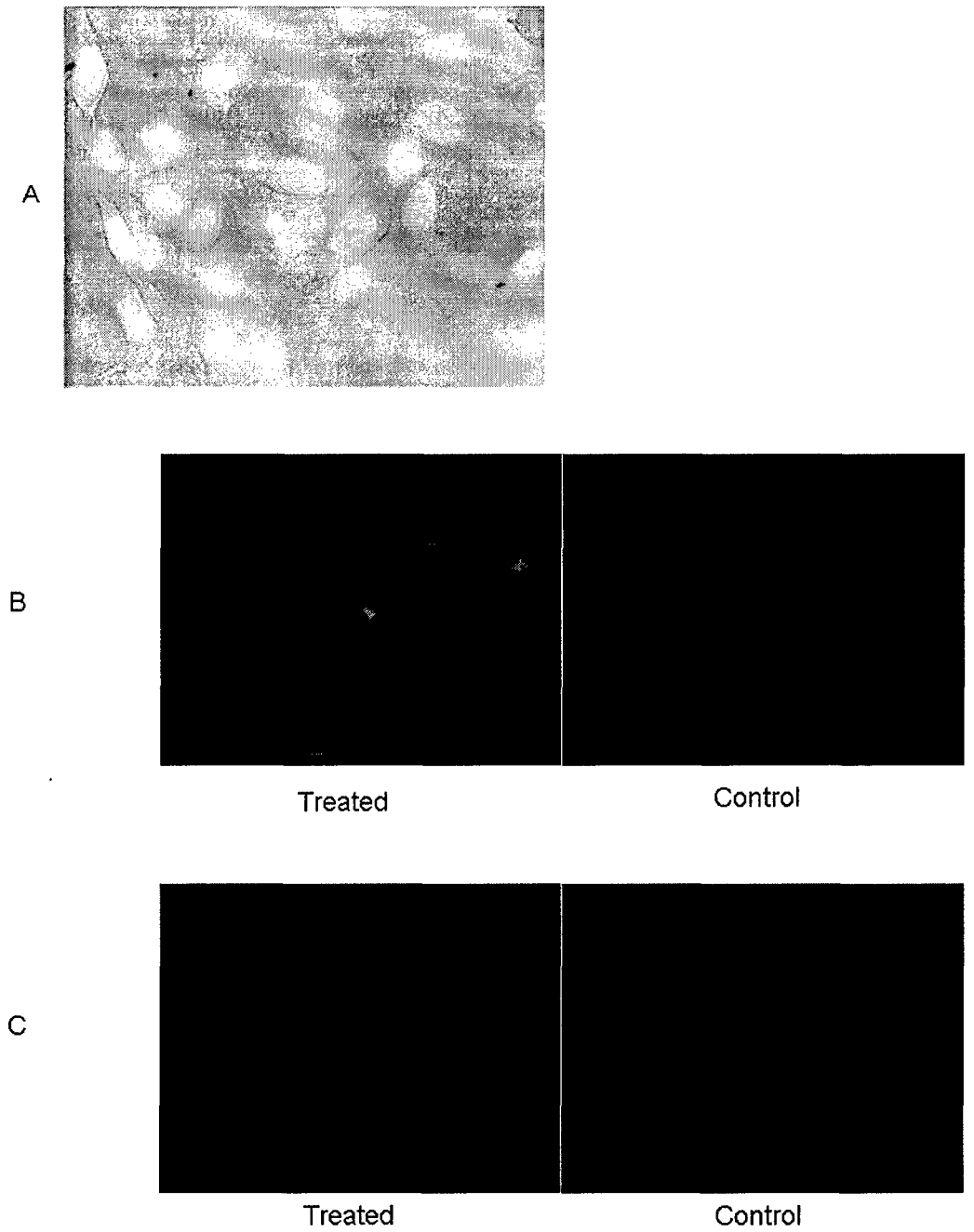


Figure 16. Immunocytochemical analysis of BT20 breast cancer cells treated with anti-3,4-EQ. **A**. Fluorescence image overlayed on brightfield image of BT20 cells treated for 5 hours with 30 μM 3,4-EQ, then fixed and treated with anti-3,4-EQ and anti-rabbit IgG conjugated to Alexa 488. **B.** Treated: fluorescence image of cells treated as in A. Control: fluorescence image of cells treated with vehicle, anti-3,4-EQ and anti-rabbit Alexa 488. **C**. DAPI nuclear stain of same fields as in **B**. Images **B** and **C** were made with the same exposure times taking care to avoid photobleaching.

Task 4. Look for TrxR adducts by immunohistochemistry in invasive breast cancer and adjacent ductal carcinoma *in situ*.

We currently have no positive controls for validation and optimization of the reagent anti-3,4-EQ for analysis of tissues. We acknowledge this flaw in the approved experimental design. However we do have a plan to address the situation. Another group created controls for a similar mass spectrometric analysis of for 3,4-EQ-derived glutathione metabolites in breast tissue by injecting 3,4-EQ into the mammary fat pads of living rats.[17] They then used mammary tissues from the sacrificed animals were to develop and validate their protocol. We will use the same strategy, but since this type of use of animals is not allowed by the funding mechanism supporting the current work, we will apply for funding from our institute to complete this Task.

Key Research Accomplishments

- Determined potency and characterized mechanism of inhibition of TrxR by endogenous electrophiles.
- Demonstrated that TrxR is alkylated by endogenous electrophiles at its C-terminus.
- Showed that C-terminally alkylated TrxR1 is both necessary and sufficient to cause the disruption of p53 conformation.
- Studied the effects of TrxR1 and TrxR2 knockdown on PG-induced production of ROS.
- Observed a selective, dose-dependent interaction between anti-3,4-EQ and proteins from breast cancer cells treated with 3,4-EQ by immunochemical (western blot) analysis of cell lysates .
- Demonstrated a selective interaction between anti-3,4-EQ and nuclear epitopes of intact breast cancer cells treated with 3,4-EQ.

Reportable Outcomes

- Portions of the work contained in this report were presented by the PI in a poster, abstract # LB-165 at the 95th annual meeting of the American Society for Cancer Research, March 27-31, 2004 in Orlando, Florida. Authors were Cassidy P, Edes K, Fitzpatrick, F and Moos P.

- Data generated in work supported by this award was included as preliminary data in a grant funded effective 1 July 2005 R01CA115616-01 by the National Institutes of Health, "The Role of Selenoproteins in the Etiology of Cancer" Philip Moos, PI. The PI on the current grant is listed as critical personnel on the new application.

- A revised manuscript reporting parts of the work in this report has been submitted to the Journal of Biological Chemistry (Appendix 1)

Personnel receiving pay from this research effort

Pamela Cassidy (PI)
Mark Wade (Research Associate)

Conclusions

We have data to support the existence of a novel gain of function for the important antioxidant enzyme thioredoxin reductase. Our data supports the hypothesis that formation of C-terminally modified TrxR causes conformational derangement of the tumor suppressor p53. This model has importance in the field of chemoprevention, specifically in efforts to use selenium supplementation,[2] and the use of NSAIDS and antioxidants for the prevention of cancer.

Anestal and Arnér[18] have shown that some forms of C-terminally modified TrxR promote apoptosis. The importance of proapoptotic properties of modified forms of TrxR produced by interactions with endogenous electrophiles are the subject of the grant noted in Reportable Outcomes and the manuscript included in. Understanding this phenomenon and how a cell might escape TrxR-mediated apoptosis will be important to our understanding of how breast cancer arises.

One reagent, anti-3,4-EQ has been identified that reacts selectively with cells treated with 3,4-EQ. Anti-3,4-EQ appears to be useful for immunochemical analysis of proteins by western blot. Anti-3,4-EQ also has potential as a reagent for immunocytochemistry. Although the concentrations of 3,4-EQ used in our experiments were at least an order of magnitude higher than estrogen levels found in normal tissue, glutathione and N-acetylcysteine deriviatives of 3,4-EQ are found in breast cancers,[19] so we believe that our reagent has possible utility in the detection of this important biomarker. In the future we will generate positive controls from rat mammary tissue for use in developing an immunohistochemical protocol for the examination of human breast tissues.

[2] Thioredoxin reductase is one of a number of selenoproteins whose levels and activity are responsive to selenium supplementation.

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Inhibition of Thioredoxin Reductase Activity by Endogenous Electrophilic Lipids, Site Directed Mutagenesis and siRNA: Active Site Modification and Selenoenzyme Depletion have Different Cellular Effects

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Running Title: Biological consequences of TrxR inactivation.

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Summary:

The covalent modification of macromolecules by exogenous and endogenous electrophiles can disrupt cellular function. One cellular target for lipid electrophiles is thioredoxin reductase. Here we report that covalently modified thioredoxin reductase results in a change of function that is functionally distinct from the depletion of thioredoxin reductase protein. A thorough kinetic analysis was performed on purified thioredoxin reductase using several classes of endogenous electrophiles to examine the mechanism of inactivation. The consequences of thioredoxin reductase alkylation on p53 conformation and cell death were then examined. We found C-terminal disruption of thioredoxin reductase is both necessary and sufficient for p53 conformational derangement and promotion of apoptosis. Using a combination of a C-terminal thioredoxin reductase mutant and small interfering RNA directed against endogenous thioredoxin reductase, we demonstrate that lipid-inactivated thioredoxin reductase is functionally equivalent to C-terminal mutant thioredoxin reductase, but not to the absence of thioredoxin reductase protein.

Thioredoxin reductase ($TrxR^1$) is a homodimeric selenoprotein that catalyses NADPH-dependent reactions (1-3). TrxR is a promiscuous enzyme (4-10) involved in the antioxidant network (11-13) and cellular proliferation (14) by providing reducing equivalents either directly or via Trx (3,15-19). The TrxR-Trx system also maintains the redox state of many transcription factors including p53, AP-1 and NF-κB (2,3,20-25).

Some electrophilic lipids with α,β unsaturated carbonyl substituents, derived from arachidonate metabolism, attenuate the activity of TrxR when cells are treated in a pharmacological manner, or when the lipids are generated endogenously by the controlled induction of 15-LOX (26,27). These lipids promote conformational and functional derangement of the tumor suppressor p53 by impairing TrxR (27).

In the current work we characterized the interactions of electrophilic lipids with purified TrxR enzyme to clarify the kinetics and mechanism of inactivation. We compared several types of endogenous electrophiles including electrophilic eicosanoids, the 5-LOX allylic epoxide metabolite, LTA_4, the lipid peroxidation product, 4-HNE, and a quinone metabolite of estrogen. We used small-interfering RNAs (siRNA) to reduce cellular TrxR1 expression and compare the effects of its depletion with the effects of its inactivation by chemically reactive lipids or site-directed mutagenesis directed at the catalytic selenocysteine. We report that the C-terminal inactivated form of TrxR1 is both necessary and sufficient for the disruption of the wild type conformation of p53, and that this altered form of TrxR1 is a mediator of electrophile-induced apoptosis. Modification of the active site selenol of TrxR is functionally distinct from loss of expression of TrxR.

EXPERIMENTAL PROCEDURES

Materials - Materials used included Dulbecco's modified essential medium (DMEM), Eagle's modified essential medium (EMEM) and supplements (GIBCO/BRL); 4-hydroxy-2-nonenal and prostaglandins (Cayman Chemicals); auranofin (ICN Biomedicals); 5,5'-dithio-bis(2-nitrobenzoic acid) (Sigma); Complete protease inhibitor mixture (Roche Molecular Biochemicals); Lipofectamine 2000 transfection reagent, Novex pre-cast polyacrylamide minigels (Invitrogen); Western Lightning enhanced chemiluminescence reagents (PerkinElmer Life Sciences); horseradish peroxidase-conjugated secondary antibodies; protein A/G PLUS-Agarose (Santa Cruz Biotechnology); Neutravidin-conjugated beads (Pierce); monoclonal antibodies directed against p53 (Ab-5 wt and Ab-3 mt, Calbiochem); polyclonal antibodies against p53 (FL-393-G, Santa Cruz Biotechnology) and TrxR (Upstate USA, Inc., and custom antibody services of the Pocono Rabbit Farm & Laboratory). The University of Utah DNA/Peptide Core synthesized peptides (CIPKKLMHQAALLG and CGLSEEKAVEKFGE) from TrxR1 conserved across many species and these were conjugated to KLH and OVA as carriers, the antigens were injected, in combination, into chicken hosts and antibodies were purified from egg yokes using Eggcellent Chicken IgY purification kits (Pierce).

Cell Culture - RKO colon cancer cells (gift of M. Meuth, Institute for Cancer Studies, University of Sheffield, Sheffield, U.K.) were maintained in DMEM at 37°C in a humidified incubator with 5% CO_2. Media was supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 μg/ml gentamycin, and 10% (vol/vol) FBS. MCF7 breast cancer cells (ATCC) were maintained similarly in EMEM supplemented with non-essential amino acids, 1 mM sodium pyruvate, 10 μg/ml bovine insulin, 10 μg/ml gentamycin, and 10% (vol/vol) FBS. The RKO–EcR cell line was generated by transfecting RKO cells with pVgRXR (Invitrogen) and selecting stable Zeocin resistant colonies.

Immunochemical analysis (Westerns) - Proteins were fractionated on 10% tris-glycine polyacrylamide gels, and then transferred to a 0.45 μm PVDF membrane. The membranes were blocked by incubation with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20, and then incubated with the appropriate primary antibody and horseradish peroxidase (HRP) conjugated secondary antibody or streptavidin-HRP. The membranes were treated with chemiluminescence reagents and exposed to autoradiography film.

In other experiments, purified TrxR was incubated with 200 μM NADPH in the presence of 60 μM biotinylated PGs, fractionated by SDS-PAGE, transferred to PVDF, and analyzed by immunochemical detection with neutravidin-HRP to localize to site of covalently modified TrxR. In certain of these experiments, auranofin (10 μM) was used as a specific inhibitor of the selenocysteine to compete with the PGs. In these experiments, the amount of TrxR was assessed using the chicken anti-TrxR antibody.

Immunoprecipitation of p53 - Cells were lysed in 250 mM sucrose, 50 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl2, 1 mM EDTA, complete™ protease inhibitor, 2 mM NaF, 2 mM sodium orthovanadate. We sonicated the lysate twice for 5 s at 4°C. After centrifugation at 13,000 × g, samples containing 200 μg of total protein were incubated for 16 h at 4°C with 1 μg of either Pab240 or Pab1620, antibodies that specifically recognizes mutant or wild-type conformations, respectively, of p53 under nondenaturing conditions (28) and 20 μl of protein A/G PLUS-Agarose in 1 ml of PBS with 0.4% Tween 20. The samples were centrifuged at 500×g for 5 min to isolate the immune complexes. The beads were washed twice with 1 ml of PBS/0.4% Tween 20. The samples were fractionated by SDS/PAGE as described above and the amount of conformationally mutant or wild type p53 in the immunoprecipitate was measured by hybridization with a separate anti-p53 polyclonal antibody (FL-393) which recognizes both the mutant and wild-type forms of the protein.

Thioredoxin reductase assays - The inhibition constants were determined in the presence of both inhibitor and substrate using a modification of the method described by Becker et al. (29) for determination of the rate of TrxR-catalyzed reduction of the disulfide substrate dithiobis-5-5'-dinitrobenzoic acid (DTNB). To a mixture of 870 μl reaction buffer (50 mM potassium phosphate

pH 7, 2 mM EDTA), 100 µl 2 mM NADPH, 1 µl inhibitor in DMSO, and 2 µl of enzyme (1.4 µg purified from rat liver as described (30)) was added 30 µl DTNB (0.1 M in DMSO). The rate of reduction of 3 mM DTNB in the presence of varied concentrations of electrophile was measured by monitoring the absorbance of the reaction mixtures at 412 nm. These data were used to calculate the IC_{50} values for each electrophile. Then using the equation $K_i = IC_{50}/(1+[S]/K_M)$ where [S] is the concentration of DTNB and K_M is the experimentally determined value of 0.20 mM for DTNB, the K_i values listed in Table 1 were calculated. To evaluate the time-dependent inactivation of TrxR by electrophiles, 0.54 µg (333 nM) TrxR in 30 µL reaction buffer (50 mM potassium phosphate pH 6.5, 2 mM EDTA) containing 300 µM NADPH and electrophile were incubated at 25°C. At various times, 2 µl of this mixture was withdrawn and diluted 50-fold into an assay mixture containing 50 mM potassium phosphate pH 6.5, 200 µM NADPH and 3 mM DTNB (15× K_M), for the determination of residual activity. Data were fitted using the data analysis program Origin (Microcal Software Inc.).

siRNA and TrxR constructs - RNA oligos that are specific for TrxR1 were designed and these were synthesized in the University of Utah DNA/Peptide Synthesis Core. The siRNA directed against TrxR1 is: (5'-AGACCACGUUACUUGGGCAdTdT-3'), the control is a scrambled sequence (5'-AGGCAAAUCACGGUGUCCUdTdT-3') that does not match any sequence in the Genbank human database for more than 16 nucleotides (31). The full-length TrxR1 construct was from ATCC (#6355551). The complete coding sequence was cloned into the ecdysone inducible vector, pIND(SP1)hydro (Invitrogen). This new construct does not contain the SECIS element required for selenoprotein synthesis. The C498S and U499S (from 5'-TGCTGA-3' to 5'-T<u>C</u>CT<u>C</u>A -3') mutants were prepared using QuikChange site directed mutagenesis procedures (Strategene). This construct was made insensitive to siRNA by introducing a silent mutation (5'-CCA<u>A</u>G<u>A</u>TAC-3', i.e. from CGT encoding Arg to AGA encoding Arg).

Caspase-3 assays - Cells were treated with electrophiles for the indicated length of time then lysed in 25 mM Hepes, pH 7.5, 5 mM EDTA, 2 mM DTT, 0.1% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate. Caspase-3 activity was measured fluorometrically using DEVD-AMC as a substrate (Peptides International) (32).

RESULTS

Electrophilic lipids covalently modify the C-terminus of TrxR - We investigated the kinetics and mechanisms of inactivation of isolated TrxR. We found that TrxR must be reduced with NADPH for PGA_1-APB to be covalently attached (Figure 1A, lane 2 vs. lane 3). We next asked if the C-terminal selenocysteine residue is the site of derivitazation. Auranofin, a thiogold compound, acts by modifying the C-terminal selenocysteine of TrxR (33). When reduced TrxR was first treated with auranofin, followed by either PGA_1-APB or 15-d-PGJ_2-APB, the modification by auranofin blocked the interaction of TrxR with PGs (Figure 1A, lane 3 v. 4 and lane 5 v. 6).

Endogenous electrophiles are potent, time-dependent inhibitors of TrxR - We performed enzyme kinetic experiments with purified TrxR to determine the potency of the inhibition by PGs and three other chemical classes of endogenous electrophiles (Figure 2). Two of these compounds, 4-HNE and 3,4-EQ, are chemically similar to the PGs in that they both contain electrophilic α,β-unsaturated carbonyls. They differ from the PGs however, in that 4-HNE contains an aldehyde, not a ketone, and 3,4-EQ, which is a quinone, is a potential substrate for reduction by TrxR (10). The third, LTA_4, is an allylic epoxide. Inhibitory activity was measured by an assay for the reduction the disulfide substrate DTNB (34). Table I shows the potency of inhibitors ranged from 15-d-PGJ_2 with a K_i = 23 nM to LTA_4 with K_i = 29 µM.

The coincidence of a biotin label with the TrxR band in the immunochemical analysis of SDS-PAGE-fractionated reaction mixtures in Figure 1 is consistent with a covalent interaction between PGs and TrxR. Covalent modification should result in a time-dependent inactivation of TrxR when the reduced enzyme is incubated with electrophiles (35) and the results shown in Figure 3 support this hypothesis. The time-dependent

inactivation of TrxR fit a first-order exponential decay model with a half-life of 11, 12 and 6 min for PGA_2, 15-d-PGJ_2 and LTA_4 methyl ester, respectively. The inactivation of TrxR by 15-d-PGJ_2 was evaluated at five different concentrations. We then calculated the apparent inactivation constant ($k_{inact} = 0.1\ sec^{-1}$) and binding constant ($K = 870\ \mu M$) from the y-and x-intercepts respectively (36) by plotting the half-life for inactivation versus the reciprocal of the inhibitor concentration (Figure 3B). Only 15-d-PGJ_2 was evaluated in this way due to the limited potency and solubility of PGA_2 and LTA_4.

The kinetics of inactivation of TrxR by 4-HNE and 3,4-EQ fit a model of bi-exponential decay, with an initial half-life of 20 s and a terminal half-life of 7 min (Figure 3C). In the absence of NADPH, time-dependent inactivation fit a model of a single exponential decay, again with a half-life of about 20 s. This suggests more than one mode of inactivation of TrxR by 4-HNE and 3,4-EQ.

We tested whether the electrophiles were substrates of TrxR by monitoring the consumption of NADPH in a mixture of the electrophile, TrxR and NADPH. Only 3,4-EQ, showed detectable activity, and this was very modest at 0.08 μmole $NADP^+$/min/mg TrxR. By comparison, reduction of DTNB by TrxR occurs at the rate of 35 μmol/min/mg. Thus, these inhibitors are not mechanism-based inhibitors (suicide substrates) (37).

Electrophilic Estrogen Quinones cause conformational disruption of p53 - One of the biological consequences of TrxR inhibition by PGs, or 4-HNE is the derangement of the conformation of the tumor suppressor p53 (27). This result, originally observed in colon cancer cells, is recapitulated in the breast cancer cell line MCF7 (Figure 4 panel A). The conformation of p53 is determined in an assay that takes advantage of conformation-specific antibodies to immunoprecipitate p53 either in a wild type or 'mutant' conformation. We used cancer cells derived from breast tissue as this was relevant to our examination of estrogen metabolite, 3,4-EQ. We found that treatment of cells with this compound also causes the disruption of p53 conformation (Figure 4 panel B).

Effects of TrxR1 siRNA on PG-induced disruption of p53 conformation - Investigations on *Trr1* regulation of p53 in yeast (38-40) and our prior results predicted that genetic reduction of TrxR1 would have the same effect as pharmacologic reduction by treatment with electrophiles (27) i.e. cells treated with TrxR1 siRNA alone or in combination with PGs should be more susceptible to conformational disruption of p53 than cells treated with PGs alone. We generated siRNA that specifically target TrxR1, lowered TrxR1 protein levels and reduced overall TrxR activity in RKO cells (Figure 5 panels A and B). A scrambled siRNA control, which did not align with other sequences in the Genbank database (using the short exact match BLAST (31)) had no effect on TrxR1 protein expression. We used these siRNAs to test the effects of TrxR1 depletion on p53 conformation. We found no difference in the amount of mutant p53 in cells treated with either TrxR1 or control siRNA (Figure 5 panel C). Figure 5 panel D shows less mutant p53 in cells treated with TrxR1 siRNA plus PGA_1 compared to cells treated with control siRNA and PGA_1. The p53 in cells with reduced levels of TrxR1 protein was less susceptible to conformational derangement after PG treatment.

Expression of TrxR1 C-terminal mutant causes the disruption of p53 conformation - We considered our mechanistic model for p53 inactivation in light of our new results and those reported by Anestål and Arnér (41). Namely, introduction of purified TrxR1, with C-terminal modifications, into lung cancer cells caused apoptosis, while introduction of full-length wild type TrxR1 did not. We hypothesized that C-terminal compromised TrxR1 might be necessary to mediate the conformational derangement of p53. To test this hypothesis directly, we used a siRNA-resistant TrxR1 construct containing both C498S and U499S mutations that inactivate the C-terminal active site. Expression of this construct was under control of the ecdysone receptor. After first depleting wild type TrxR1 protein by treatment with siRNA, we transfected the cells with the mutant construct, induced expression of the mutant TrxR1, and analyzed p53 conformation after 24 h. siRNA treatment suppressed expression of endogenous TrxR1. Ponasterone A treatment induced expression of the C498S, U499S mutant TrxR1

(Figure 6 panel A). Induction of mutant TrxR1 was accompanied by an increase in the amount of conformationally mutant p53 (Figure 6, panel B). Thus C-terminal modified TrxR1 is sufficient for the formation of conformationally deranged p53 but depletion of TrxR does not recapitulate this effect.

TrxR1 siRNA inhibits PG-induced apoptosis - Using TrxR1 siRNA, we investigated the role of TrxR1 in PG-induced apoptosis. Figure 7A shows the time course of effector caspase activation by 15-d-PGJ2 in RKO cells treated with TrxR1 or control siRNA. In cells treated with the control siRNA, caspase activation is maximal after 12 hours of PG treatment and then begins to decline. The increase in caspase activity is much slower in cells treated with TrxR1 siRNA; activity is still increasing at 24 hours, but has not reached the level observed with cells treated with control (scrambled) siRNA. Immunochemical analysis of TrxR1 protein from all treatments showed reduced levels in cells treated with TrxR1 siRNA compared to control siRNA (data not shown). Figure 7 panel B shows the result of caspase assays done in TrxR1-silenced cells treated with PGA_1, 4-HNE and 3,4-EQ in addition to 15-d-PGJ_2. Consistent with the data for TrxR inhibition in Table 1, 15-d-PGJ_2 is more potent than PGA_1. 4-HNE and 3,4-EQ are less efficacious than the PGs in this experiment; however 3,4-EQ is at a lower dose (32 μM for 3,4-EQ and 60 μM for the other compounds). Importantly, we observed that decreased expression of TrxR1 attenuated caspase-3 activation by all of these endogenous electrophiles.

DISCUSSION

Mammalian TrxRs are flavoenzymes which contain a C-terminal cysteine-selenocysteine active site that are responsible for the reduction of their cognate substrate thioredoxin (42). The C-terminal selenocysteine is especially vulnerable to electrophilic and oxidative modification (43). The high reactivity of this residue is a result of the low pK_a (~5.5) of the selenol group, which causes selenocysteine to be fully ionized at physiological pH. Data shown in Figures 1 and 3 support the conclusion that the reduced selenol form of TrxR is required for the reaction of the enzyme with electrophilic arachidonic acid metabolites.

We examined LTA_4, another arachidonic acid metabolite produced by 5-LOX, and 3,4-EQ, a metabolite produced by the hydroxylation and subsequent oxidation of estrogen by cytochrome P450 (44). We report that electrophilic estrogens, as well as the arachidonic acid metabolites, are potent, time-dependent irreversible inhibitors of TrxR *in vitro*.

Our kinetic data suggest that all of the lipid electrophiles share a common mechanism for modification of TrxR, which requires its C-terminal active site in a reduced form. 3,4-EQ and 4-HNE have a second, separate effect. One mode has a very short half-life of inactivation, which does not involve the reduced enzyme but still results in the irreversible disruption of the active site, perhaps by Schiff-base formation with His, Arg or Lys. The second mode involves interactions with the reduced enzyme and is similar to that observed with the PGs and LTA_4

In the strictest sense these electrophilic lipids are not affinity labels or active-site-directed irreversible inhibitors as defined by Fersht (45), because they do not resemble any known substrate of TrxR. Nonetheless these compounds do react covalently with TrxR, their rates of inactivation are decreased by substrate (DTNB) and their inhibition follows saturation kinetics. Other features of affinity labels such as pH dependence of inhibitor and substrate, and moles of inhibitor bound/mole of active sites have not been evaluated for the TrxR/electrophilic lipid systems. However we can infer from the data for the time-dependent inactivation of TrxR by 4-HNE and 3,4-EQ, that the ratio of inhibitor/mole active site could be greater than one for these compounds because of the bi-exponantial decay of activity observed.

Individual eicosanoids with α,β-unsaturated ketone substituents like PGA_1 or PGA_2 may not occur in micromolar concentrations at a site of inflammation, but inflammatory exudate contains a blend of electrophiles typified by α,β-unsaturated aldehydes derived from eicosanoid biosynthesis or lipid peroxidation (4-HNE); α,β-unsaturated ketones derived from eicosanoid metabolism (15-keto-$PGF_{2\alpha}$, 15-keto-PGE_2, 5-, 12-, and 15-oxo-ETE); and, α,β-unsaturated ketones

derived from albumin-catalyzed dehydration of PGE_2 and PGD_2 (PGA_2, Δ12-PGJ_2 and 15d-PGJ_2 (47-50). Indeed, studies have indicated that particular J-series cyclopentenone PGs are present during resolution phases of inflammation at ~1-10 μM concentrations and can contribute to the attenuation of the inflammatory response (47). Furthermore, the level of endogenous 4-HNE in tissues ranges from 0.1 to 3.0 μM and increases to ~10 μM in conditions of oxidative stress (51). Thus, inflammation likely exposes cells to a mixture of electrophiles in quantities sufficient to affect TrxR. Here we have expanded the scope of endogenous electrophiles examined to include LTA_4, another arachidonic acid metabolite produced by 5-lipoxygenase, and 3,4-EQ, a metabolite produced by the hydroxylation and subsequent oxidatation of estrogen by cytochrome P450 (44). Recently, Rogan and colleagues showed that levels of 4-catechol estrogens and their quinone conjugates were highly significant predictors of breast and other cancers (46,52).

We also examined two important biological consequences of the interaction of electrophiles with TrxR1; the conformational disruption of p53 and the induction of apoptosis. We show that electrophiles disrupt the conformation of p53 in breast cancer cells in a manner similar to that observed for colon cancer cells. Thus, the process appears to be general from a chemical and cellular perspective.

Anestål and Arnér determined that C-terminal inactivated TrxR1 proteins have distinct activity compared to the authentic wild type species containing unmodified selenocysteine (41). Using *BioPORTER* to introduce proteins directly into lung cancer cells, they found that there was an increase in apoptosis with selenium-compromised TrxR1s and that the susceptibility to apoptosis was increased markedly compared to cells treated with wild type TrxR1. Our experiments in which we transfected cells with TrxR1 mutants and/or with TrxR1 siRNA, showed that selenium-compromised TrxR1 is a critical mediator of the biological effects of endogenous electrophiles. These effects include the conformational derangement of p53 as well as induction of apoptosis.

Notably, the effect of electrophilic lipids depends on the expression of TrxR1. In other words, loss of expression of TrxR in cells has a negligible effect on p53 conformation and function. Chemical or mutational modification of the C-terminal active site of Trxr1 has a pernicious effect, i.e. the inactivation of the transcriptional functions of p53.

When TrxR is treated with the electrophilic compound 2,4-dinitrochlorobenzene (DNCB), the selenocysteine residue is modified and the resulting C-terminal nitroarylated enzyme produces superoxide in the presence of NADPH and oxygen (53). We were able to reproduce this result, but when we treated TrxR with PGs, 4-HNE or 3,4-estrogen quinone, we found no evidence of superoxide production *in vitro* (data not shown). Consequently, we do not believe that TrxR1 is directly involved in PG-induced production of ROS (54,55). Consistent with our contention is the work of Nordberg et al. (17) who showed that while many covalent modifications of the C-terminal selenocysteine of TrxR1 inhibited normal enzyme activity, only modifications containing an aromatic nitro group were able to produce superoxide. They speculated that the peculiar redox properties of the nitro group allowed this chemical moiety to shuttle single electrons from the N-terminal thiol-flavin charge transfer complex of TrxR to oxygen in solution resulting in the production of superoxide.

We have presented data from experiments that exploit the genetic depletion of TrxR1 to investigate the role of this selenoprotein in the inactivation of the tumor suppressor p53 and the induction of apoptosis by endogenous electrophiles. Our initial hypothesis that depletion of TrxR1 would potentiate the conformational derangement of redox-sensitive p53 in electrophile-treated cells due to depletion of reduced Trx, was based on the study by Merwin et al. (40). They showed that reporter gene activity of human p53 was compromised in *Trr1* null yeast. We found that reduction of TrxR1 levels protected p53 conformation in colon cancer cells treated with endogenous electrophiles. We also found that reduced TrxR1 levels antagonized electrophile-induced apoptosis. Our data is consistent with the observations of Anestål and Arnér (41) and support a model in which C-terminal modified TrxR1 is the species through which two important biological activities of endogenous electrophiles

are manifest. A model of this gain-of-function role for modified TrxR is illustrated in Figure 8. The new function(s) of chemically and mutationally modified TrxR is currently under investigation. TrxR synthesis and activity are directly related to selenium levels (56,57). Thus, the observation that selenium sufficiency increases both TrxR activity and spares p53 function in cells treated with electrophilic lipids (27) provides an explicit mechanistic framework in which to understand how dietary selenium confers protection against cancer.

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FOOTNOTES

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[1] The abbreviations used are: DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid); 15-d-PGJ_2: 15-deoxy-Δ-12,14-PGJ_2; 15-d-PGJ_2-APB: 15-deoxy-Δ-12,14-PGJ_2-aminopentylbiotin; 3,4-EQ: 3,4-estronequinone; 4-HNE: 4-hydroxynonenal; LOX: lipoxygenase; LTA_4: leukotriene A_4; PG: prostaglandin; PGA: prostaglandin A; PGA_1-APB:prostaglandin A_1-aminopentylbiotin; siRNA: small interfering RNA; Trx: thioredoxin; TrxR: thioredoxin reductase; PVDF: polyvinylidenedifluoride

Table 1. Inhibition of TrxR by endogenous electrophiles.

Compound	IC50 (μM)	K_i (μM)
15-d-PGJ_2	0.36 ±0.04	0.024 ±0.003
PGA_2	68 ±9	4.5 ±0.6
4-HNE	12 ±2	0.8 ±0.1
LTA_4 methyl ester	513 ±87	33 ±6
3,4-EQ	20 ±6	1.0 ±0.3

The inhibition constants were determined in the presence of both inhibitor and substrate using a modification of the method described by Becker et al. (29) for determination of the rate of TrxR-catalyzed reduction of the disulfide substrate DTNB as described in the Experimental Procedures.

FIGURE LEGENDS

Figure 1. Electrophiles alkylate the C-terminus of TrxR. Purified TrxR was incubated with 200 μM NADPH in the presence of 60 μM 15-d-PGJ_2-APB or PGA_1-APB followed by fractionation by SDS-PAGE and immunochemical detection with neutravidin-HRP to localize covalently modified TrxR, containing a biotin epitope (lanes 3 and 5). In lane 2, NADPH was omitted from the mixture to illustrate the requirement for reduction of the enzyme. Auranofin (10 μM), which specifically interacts with the C-terminus of TrxR, antagonized the reaction with 15-d-PGJ_2-APB or PGA_1-APB (lanes 4 and 6).

Figure 2. Electrophilic lipids. Arrows point to site(s) of electrophilic attack. 4-HNE and 3,4-EQ are susceptible to both 1,4- and 1,2-reactions.

Figure 3. Time-dependent inactivation of TrxR by 15-d-PGJ_2 and other electrophiles. A: The rate of reduction of DTNB by TrxR was measured following incubation with PGA_1, 15-d-PGJ_2, or LTA_4-methylester for 0-40 min. These electrophiles demonstrate first-order kinetics. B: The half-time of inhibition of TrxR at various concentrations of 15-d-PGJ_2 was plotted as a function of 1/[15-d-PGJ_2]. Analysis of the resulting line gives k_{inact} and K_I for the covalent reaction between the PG and TrxR (36). C: 3,4 –EQ and 4-HNE were evaluated as in A. Data fit an equation described by 2 exponential decays one with a half-life of 20 s, the other with a half-life of 7 minutes. When NADPH was left out of the pre-incubation mixture (circles and dotted line), the data fit an equation with a single exponential decay with half-life of inactivation of 20 s.

Figure 4. Endogenous electrophiles cause disruption of wild-type p53 conformation. A: MCF7 breast cancer cells were treated with PGA_2 or 15-d-PGJ_2 for six h. at the concentrations specified and accumulated more p53 protein in mutant (Pab 240 epitope) conformation. Cells treated with PGs had more mutant p53 than those treated with vehicle. B: MCF7 cells were treated with 3,4-EQ for 5 h. Analysis of p53 conformation as in panel A demonstrated that 3,4-EQ treated cells had more mutant p53 than did cells treated with vehicle alone.

Figure 5. TrxR1 siRNA lowers TrxR1 protein levels, enzymatic activity and makes p53 <u>less</u> susceptible to PG-induced conformational derangement. A. RKO colon cancer cells were treated with 20 nM siRNA directed against TrxR1 or with a control, scrambled siRNA. The cells were lysed after the indicated times and protein was analyzed for TrxR1 by immunoblot. TrxR1 protein levels were reduced ~75% after 72 h incubation. B. Enzymatic analysis of protein from cells treated with TrxR1 siRNA showed a ~70% reduction in TrxR activity compared to control. C. RKO cells were treated with TrxR1 and control siRNAs, then the amount of p53 protein in mutant and wild type conformation was analyzed immunochemically. TrxR1 siRNA alone had no effect on p53 conformation. D. RKO cells were treated with TrxR1 or control siRNA for 48 h, then with the indicated concentrations of PGA_1 for six h. Analysis of mutant p53 showed that the protein in cells pre-treated with TrxR1 siRNA was less susceptible to conformational derangement than p53 from cells treated with scrambled siRNA.

Figure 6. Expression of C-terminal mutant TrxR1 causes conformational disruption of p53. A: RKO-ECR cells were transfected with siRNA directed against TrxR1 and a construct, pIND(SP1) TrxR CU-SS, that encodes a ponasterone-inducible catalytically inactive C-terminal mutant TrxR1, that is also resistant to siRNA. Protein from cells treated with ponasterone and vehicle was fractionated by SDS-PAGE and analyzed for TrxR1 by immunoblot. In the absence of ponasterone, TrxR1 protein was depleted. Robust expression of the mutant CU-SS TrxR1 protein was detected in from ponasterone-treated cells. B: Conformational analysis of p53 protein from cells transfected with TrxR1 siRNA and the pIND(SP1) TrxR CU-SS construct show that expression of C-terminal mutated TrxR1 is sufficient for disruption of p53 conformation.

Figure 7. siRNA directed against TrxR1 affects electrophile-induced caspase activation. A: RKO cells were transfected with TrxR1 siRNA or control siRNA. After 24 h, cells were treated for the indicated time with 60 μM 15-d-PGJ_2. Cells were then harvested and analyzed for caspase 3. B:RKO cells were transfected with TrxR1 siRNA or control siRNA for 24 h, then treated with

for 20 h, then treated with: 15-d-PGJ_2 and PGA_1 60 μM; and 3,4-EQ 32 μM. In all cases, caspase 3 activity was lower in TrxR1 siRNA-treated cells compared to those treated with control siRNA.

Figure 8. Gain-of-function model for the biological activity of C-terminal-modified TrxR1. Unmodified TrxR1 is a homodimer. The N-terminal active site contains two cysteines that are reduced by NADPH through an FAD cofactor. The N-terminal active site of one subunit then transfers reducing equivalents to the C-terminal cysteine-selenocysteine redox-pair of the other subunit. This reduced form of the unmodified enzyme is able to reduce the substrate Trx and is susceptible to covalent modification by cyclopentenone PGs or other electrophiles. C-terminal modified TrxR1 is unable to reduce Trx, but has new functions. Modified TrxR1 mediates the conformational disruption of p53 and PG-induced apoptosis via activation of caspase 3.

Figure 1:

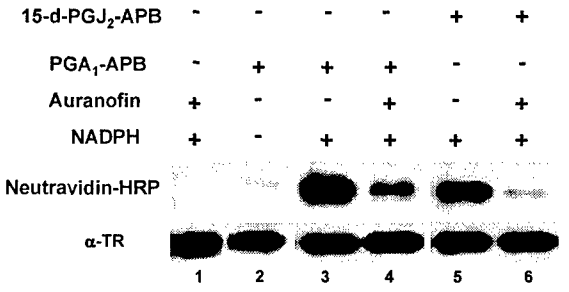

15-d-PGJ2-APB - - - - + +
PGA1-APB - + + + - -
Auranofin + - - + - +
NADPH + - + + + +
Neutravidin-HRP
α-TR
1 2 3 4 5 6

Figure 2:

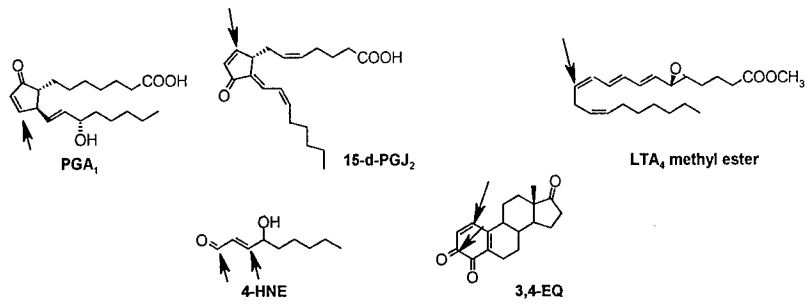

COOH
OH
PGA1
COOH
15-d-PGJ2
COOCH3
LTA4 methyl ester
OH
4-HNE
3,4-EQ

Figure 3:

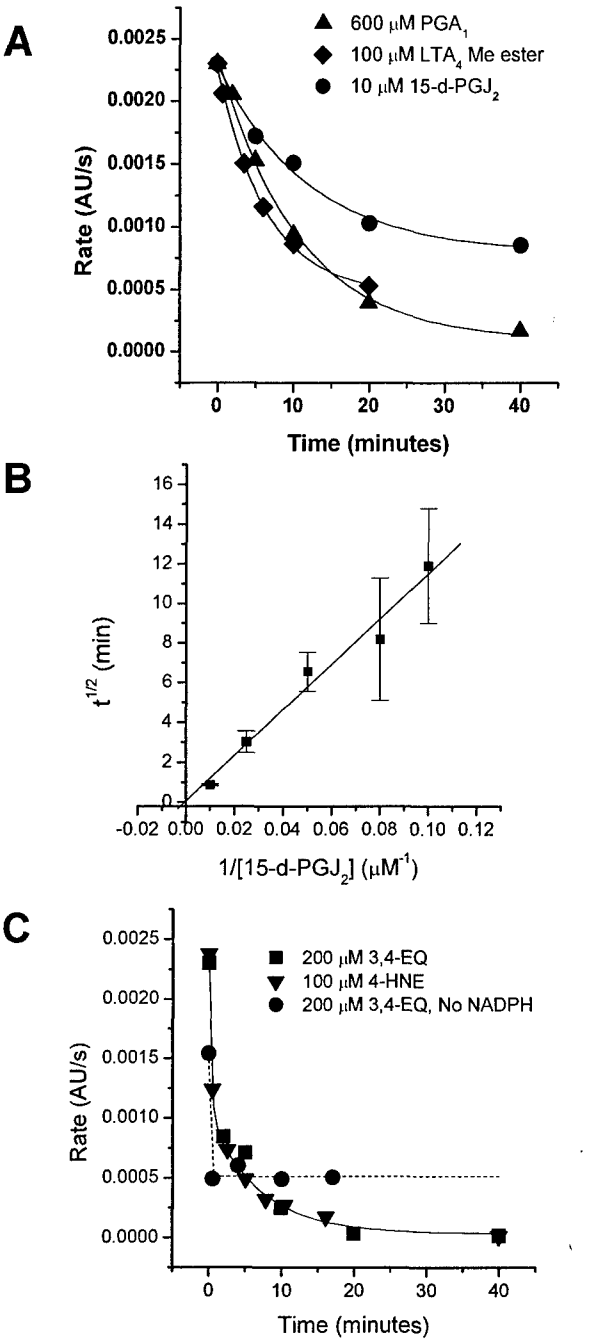

A
600 μM PGA1
100 μM LTA4 Me ester
10 μM 15-d-PGJ2
Rate (AU/s)
Time (minutes)
B
t1/2 (min)
1/[15-d-PGJ2] (μM-1)
C
200 μM 3,4-EQ
100 μM 4-HNE
200 μM 3,4-EQ, No NADPH
Rate (AU/s)
Time (minutes)

Figure 4:

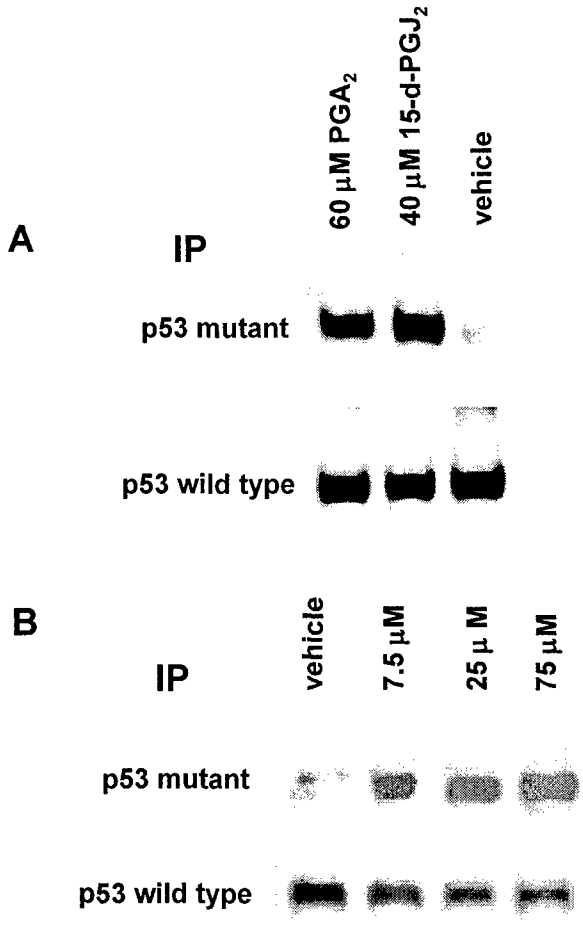

A
IP
60 μM PGA2
40 μM 15-d-PGJ2
vehicle
p53 mutant
p53 wild type
B
IP
vehicle
7.5 μM
25 μ M
75 μM
p53 mutant
p53 wild type

Figure 5:

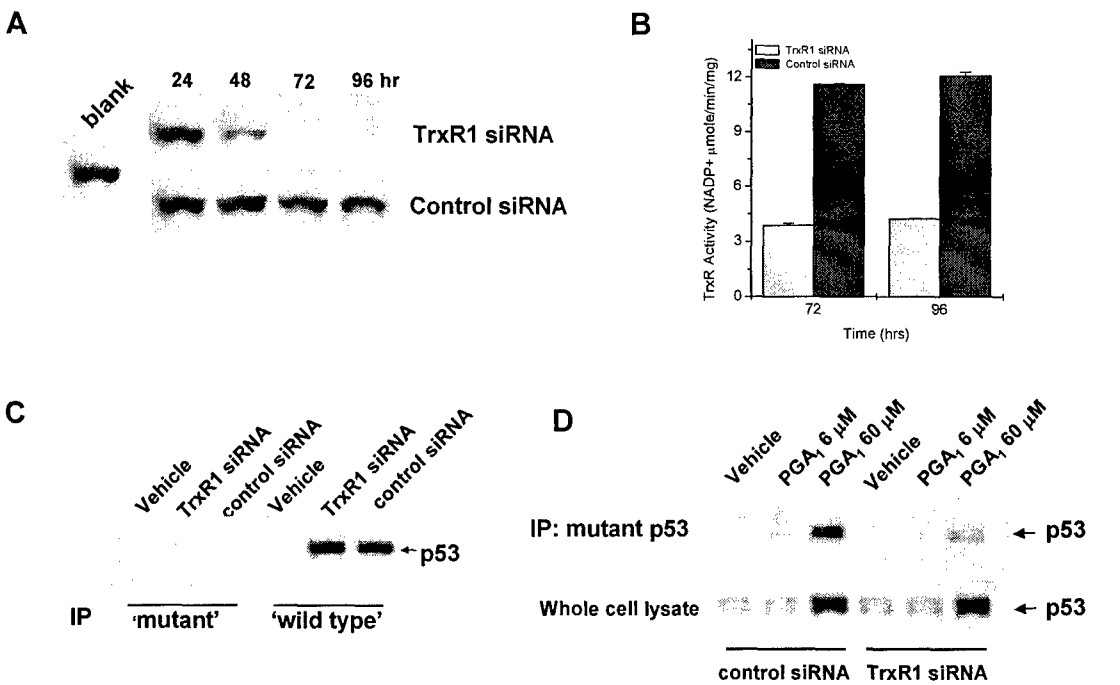

A
blank
24
48
72
96 hr
TrxR1 siRNA
Control siRNA
B
TrxR1 siRNA
Control siRNA
TrxR Activity (NADP+ µmole/min/mg)
12
9
6
3
0
72
96
Time (hrs)
C
Vehicle
TrxR1 siRNA
control siRNA
Vehicle
TrxR1 siRNA
control siRNA
p53
IP
'mutant'
'wild type'
D
Vehicle
PGA1 6 µM
PGA1 60 µM
Vehicle
PGA1 6 µM
PGA1 60 µM
IP: mutant p53
p53
Whole cell lysate
p53
control siRNA
TrxR1 siRNA

Figure 6:

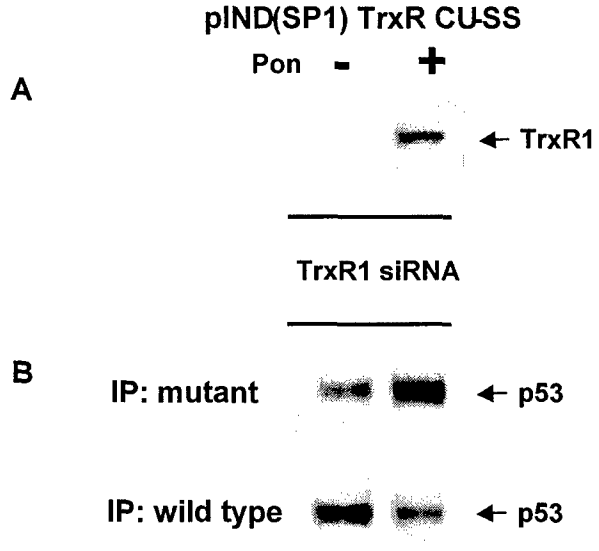

pIND(SP1) TrxR CUSS
Pon - +
A
← TrxR1
TrxR1 siRNA
B
IP: mutant
← p53
IP: wild type
← p53

Figure 7:

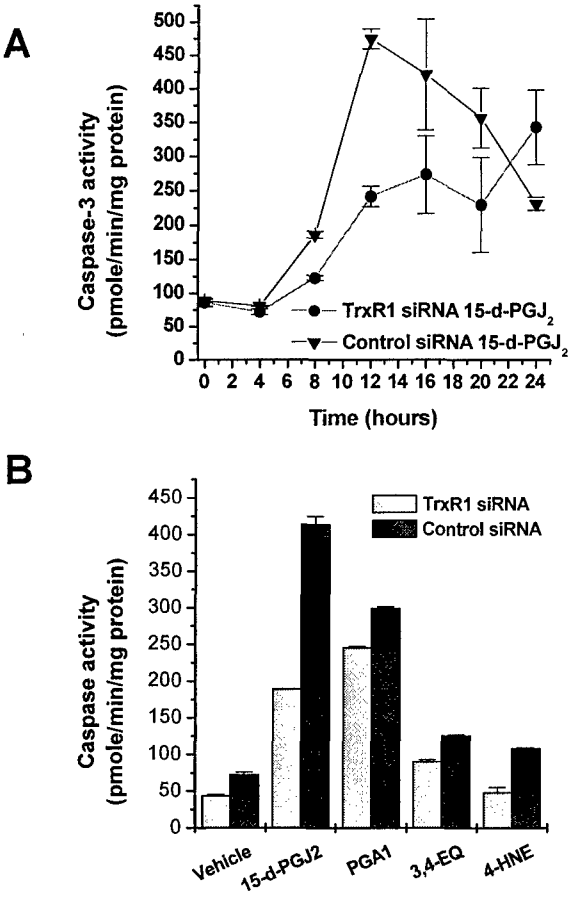
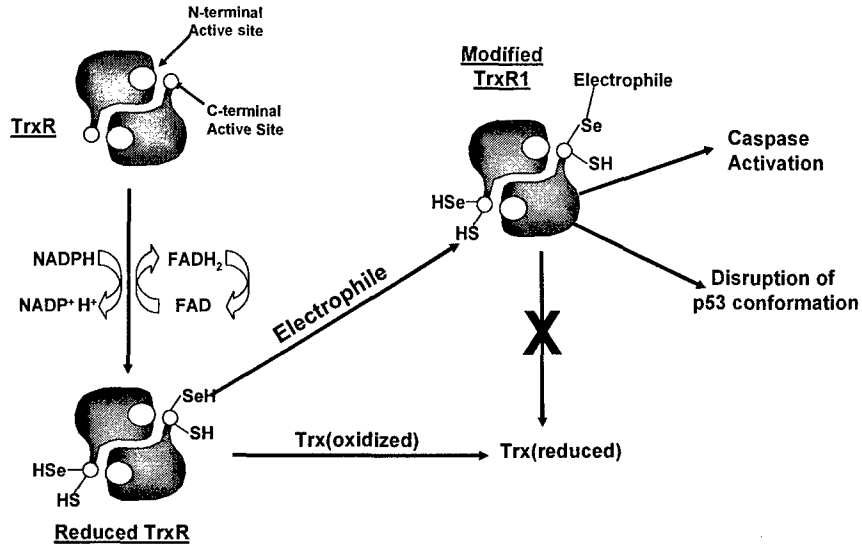

A
Caspase-3 activity
(pmole/min/mg protein)
500
450
400
350
300
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (hours)
TrxR1 siRNA 15-d-PGJ2
Control siRNA 15-d-PGJ2
B
Caspase activity
(pmole/min/mg protein)
450
400
350
300
250
200
150
100
50
0
TrxR1 siRNA
Control siRNA
Vehicle
15-d-PGJ2
PGA1
3,4-EQ
4-HNE

Figure 8:


N-terminal
Active site
C-terminal
Active Site
TrxR
NADPH
NADP+ H+
FADH2
FAD
SeH
SH
HSe
HS
Reduced TrxR
Electrophile
Trx(oxidized)
Trx(reduced)
Modified
TrxR1
Electrophile
Se
SH
HSe
HS
Caspase
Activation
Disruption of
p53 conformation