Effects of Selenoprotein X Gene Silencing and Hydrogen Peroxide Treatment on Selenoprotein Gene Expression and Antioxidant Enzyme Activity in Normal Human Liver Cells (Postprint)

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Abstract

This study aimed to investigate the effects of selenoprotein X (SelX) gene silencing and hydrogen peroxide (H2O2) treatment on selenoprotein gene expression and antioxidant enzyme activity in human normal liver cells (LO2), thereby laying a foundation for exploring the regulatory relationship between SelX and other selenoproteins as well as the function of SelX. The LO2 cell line with stable SelX gene silencing (siSelX) and the control cell line (CK) preserved in our laboratory were used to compare their growth rates by the thiazolyl blue (MTT) assay; after treatment of siSelX and CK with 200 mol/L H2O2 for 2.5 h, real-time fluorescence quantitative PCR was employed to examine the expression levels of 25 selenoprotein genes; simultaneously, the effects of SelX gene silencing and H2O2 treatment on glutathione peroxidase (GSH-Px) activity, superoxide dismutase (SOD) activity, and total antioxidant capacity (T-AOC) in the LO2 cell line were compared. The results showed that: 1) SelX gene silencing significantly reduced the growth rate of the LO2 cell line ($P<0.05$); 2) SelX gene silencing significantly upregulated the expression levels of Gpx6, Txnrd2, and Seli genes ($P<0.05$), and significantly downregulated the expression levels of Dio2, Dio3, Selm, and Sepw1 genes ($P<0.05$); H2O2 treatment significantly upregulated the expression level of Dio1 gene ($P<0.05$), and significantly downregulated the expression level of Gpx2 gene ($P<0.05$); H2O2 treatment after SelX gene silencing significantly upregulated the expression levels of Gpx6, Txnrd2, and Dio1 genes ($P<0.05$), and significantly downregulated the expression levels of Txnrd3, Dio2, Selm, Sepw1, and Selv genes ($P<0.05$); 3) SelX gene silencing significantly increased GSH-Px activity in the LO2 cell line ($P<0.05$), and significantly increased SOD activity ($P<0.05$); H2O2 treatment after SelX gene silencing significantly increased GSH-Px activity ($P<0.05$). These results

indicate that SelX gene silencing reduced the growth rate of the LO2 cell line, H2O2 treatment after SelX gene silencing affected the expression levels of some selenoprotein genes in the cells, and also influenced GSH-Px and SOD activities.

Full Text

Effects of Selenoprotein X Knockdown and Hydrogen Peroxide Challenge on Selenoprotein Gene Expression and Antioxidant Enzyme Activities in Human Normal Hepatocytes

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Abstract

This study investigated the effects of selenoprotein X (SelX) gene knockdown and hydrogen peroxide (H O) challenge on selenoprotein gene expression and antioxidant enzyme activities in human normal hepatocyte (LO2) cells to elucidate the regulatory relationships between SelX and other selenoproteins and to establish a foundation for exploring SelX function. Using SelX-stable knockdown LO2 cell lines (siSelX) and control cell lines (CK) maintained in our laboratory, we compared their growth rates using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. After treating siSelX and CK cells with 200 mol/L H O for 2.5 h, we examined the expression levels of 25 selenoprotein genes via quantitative real-time PCR. Additionally, we assessed the effects of SelX knockdown and H O treatment on glutathione peroxidase (GSH-Px) activity, superoxide dismutase (SOD) activity, and total antioxidant capacity (T-AOC) in LO2 cells. The results demonstrated that: (1) SelX knockdown significantly reduced the growth rate of LO2 cells ($P<0.05$); (2) SelX knockdown significantly upregulated Gpx6, Txnrd2, and Seli gene expression ($P<0.05$) while downregulating Dio2, Dio3, Selm, and Sepw1 gene expression ($P<0.05$). H O treatment alone significantly upregulated Dio1 expression ($P<0.05$) and downregulated Gpx2 expression ($P<0.05$). Combined SelX knockdown and H O treatment significantly upregulated Gpx6, Txnrd2, and Dio1 expression ($P<0.05$) and downregulated Txnrd3, Dio2, Selm, Sepw1, and Selv expression ($P<0.05$); (3) SelX knockdown significantly increased GSH-Px and SOD activities in LO2 cells ($P<0.05$). Combined SelX knockdown and H O treatment significantly enhanced GSH-Px activity ($P<0.05$). These findings indicate that SelX knockdown reduces LO2 cell growth rate, influences the expression of other selenoprotein genes when combined with H O challenge, and modulates GSH-Px and SOD activities.

Keywords: SelX gene; knockdown; H O ; expression

Selenium (Se) is an essential trace element for mammalian metabolism that plays crucial roles in animal growth and reproduction. In animals, Se primarily exerts its physiological functions through selenoproteins, with 25 selenoproteins identified in humans to date. Many selenoproteins serve as critical components of the cellular antioxidant defense system, scavenging oxygen free radicals and protecting biological membrane integrity. Dietary Se supplementation can enhance selenoprotein expression and improve immune and antioxidant functions. However, the functions and mechanisms of many selenoproteins remain unclear, including selenoprotein X (SelX). Also known as selenoprotein R (SelR) and methionine sulfoxide reductase B1 (MsrB1), SelX is widely distributed in animals and belongs to the methionine sulfoxide reductase (Msr) family, localizing to both the nucleus and cytoplasm. Msr is an oxidoreductase repair enzyme that restores function to oxidized ribosomal proteins. Excessive reactive oxygen species (ROS) production or incomplete clearance causes oxidative damage. To combat this, organisms possess defense systems, including Msr as both a repair enzyme and indirect ROS scavenger. Methionine is among the most readily oxidized amino acid residues in proteins, susceptible to oxidation by H O , hydroxyl radicals, sodium hypochlorite, and peroxynitrite (ONOO) to form methionine sulfoxide (MetO). Like other Msr enzymes, SelX reduces MetO back to methionine, protecting against oxidative damage and inhibiting ONOO -induced apoptosis. Previous studies show that SelX knockdown significantly increases ROS levels in human lens epithelial cells (hLE), activating protective mechanisms. Our laboratory has found that SelX knockdown reduces viability and increases apoptosis in human normal hepatocytes (LO2).

Selenoproteins incorporate Se as selenocysteine (Sec), typically located at the active site. Sec is encoded by the UGA codon, normally a stop codon in protein synthesis, with Sec insertion dependent on the SECIS (selenocysteine insertion sequence) stem-loop structure in the selenoprotein mRNA 3' -untranslated region (3' -UTR). This unique SECIS structure enables gene silencing via RNA interference targeting the 3' -UTR, a strategy used to investigate selenoprotein function in studies of GPX4, TR1, SelW, and Sep15.

Twenty-five selenoprotein genes are known in humans and mammals, many maintaining redox balance, but potential functional interactions remain unclear. The impact of SelX knockdown on other selenoprotein genes is unreported. Our previous work showed high SelX expression in liver and kidney tissues. We previously constructed SelX-knockdown LO2 cells (siSelX) and empty vector control cells (CK). This study examined SelX knockdown effects on LO2 cell growth rate and morphology, compared its impact on 25 selenoprotein mRNA expression levels and H O challenge responses, and assessed GSH-Px, SOD, and T-AOC activities. These results will provide insights into regulatory relationships between SelX and other selenoproteins, establish a foundation for understanding SelX biological functions, and offer clues about how selenoproteins mediate Se nutritional functions.

1.1 Major Equipment

CO incubator (Thermo), biosafety cabinet (Thermo), microplate reader (Molecular Devices), 5804R refrigerated centrifuge (Eppendorf), Milli-Q Plus ultrapure water system (Millipore), Nanodrop microvolume nucleic acid analyzer (Thermo), 7900HT real-time PCR system (ABI), inverted fluorescence microscope (Nikon TS100).

1.2 Major Reagents and Cell Lines

Reagents: DMEM/F12 basal medium, fetal bovine serum, and TRIzol reagent (all from Thermo Fisher); PrimeScript™ RT reagent kit, Taq DNA polymerase, and SYBR Green Mix (all from Takara Bio); primers synthesized by Sangon Biotech; GSH-Px, SOD, and T-AOC assay kits (all from Nanjing Jiancheng Bioengineering Institute).

Cell lines: siSelX and CK cells were constructed and maintained in our laboratory.

1.3 Culture of siSelX and CK Cells

siSelX and CK cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 600 g/mL hygromycin B at 37°C with 5% CO . Medium was changed every 2 days. Cells were passaged with 0.25% trypsin upon reaching 90% confluence.

1.4 Comparison of Growth Rates Between siSelX and CK Cells

Cell concentrations were adjusted to approximately 1×10 cells/mL using a hemocytometer, then seeded at 200 L/well in 96-well plates. The two outer wells in each row served as blank controls containing only medium, while the remaining 10 wells contained siSelX or CK cells (n=5 each), with this pattern repeated across 7 rows. After seeding, plates were incubated at 37°C with 5% CO , with medium changes every 2 days. At 24-hour intervals, one row was processed by removing medium, adding 180 L/well fresh basal medium, then adding 20 L/well MTT solution (5 mg/mL) and incubating for 4 h. After incubation, medium was carefully removed, 150 L/well DMSO was added, and plates were agitated to dissolve formazan crystals. The solution was transferred to a new 96-well plate, and absorbance at 570 nm was measured (blank-subtracted) to generate growth curves.

1.5 Morphological Observation of siSelX and CK Cells Before and After H O Treatment

siSelX and CK cells were seeded in separate 6-well plates and cultured at 37°C with 5% CO , with medium changes every 2 days. When cells reached 70% confluence, morphological features were observed microscopically. Cells were then treated with 200 mol/L H O for 2.5 h and observed again.

1.6 Quantitative Analysis of Selenoprotein Gene Expression

Cell concentrations were adjusted to 2×10 cells/mL and seeded at 0.5 mL/well in 24-well plates (2 plates per cell line). After incubation at 37°C with 5% CO and medium changes every 2 days, cells at 90% confluence were treated with complete medium containing 0 or 200 mol/L H O for 2.5 h. Cells were collected by washing with cold PBS, adding 200 L TRIzol reagent per well, and combining 4 wells into one sample (n=6). Total RNA was extracted using TRIzol, quality-checked via agarose electrophoresis and spectrophotometry, then adjusted to 200 ng/ L for cDNA synthesis using the RT kit.

Sequences for 25 selenoprotein genes and 2 housekeeping genes [-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were downloaded from NCBI GenBank. Primers were designed using ABI Primer Express 3.0 and synthesized by Sangon Biotech (sequences in Table 1). Real-time PCR reactions (10 L) contained 5.0 L 2×SYBR Green Mix, 0.5 L cDNA, 1.5 L primer mix (2 mol/L), and 3.0 L H O, run in duplicate. Cycling conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 34 s, followed by melt curve analysis (95°C for 15 s, 60°C for 60 s, 95°C for 15 s). Relative expression was calculated using the ΔΔCt method: ΔCt = Ct_target - Ct_reference, with CK expression set as 1 (ΔCt_R). ΔΔCt = ΔCt_target - ΔCt_R, yielding relative expression = 2^(-ΔΔCt).

1.7 Analysis of Cellular Antioxidant Enzyme Activities

Cells were seeded at 2×10 cells/mL (2 mL/well) in 6-well plates (8 plates per cell line). After culture at 37°C with 5% CO and medium changes every 2 days, 90% confluent cells were treated with 0 or 200 mol/L H O for 2.5 h. Cells were collected by washing twice with cold PBS, adding 150 L PBS per well, and scraping. Four wells were combined per sample (n=6). Cells were sonicated to release proteins, centrifuged, and supernatants were assayed for GSH-Px, SOD, and T-AOC activities following kit protocols.

1.8 Statistical Analysis

Data were analyzed using SPSS Statistics 13.0, presented as means ± SEM. Growth rates were compared by independent samples t-test. Selenoprotein gene expression and enzyme activities were analyzed by one-way ANOVA with Duncan' s multiple comparison test. $P<0.05$ was considered significant.

2 Results and Analysis

2.1 Growth Rate Analysis of siSelX and CK Cells

Mitochondrial succinate dehydrogenase in viable cells reduces MTT to insoluble purple formazan crystals, while dead cells lack this activity. DMSO dissolves these crystals, producing a characteristic absorbance peak at 570 nm that indirectly reflects cell number. Growth rates were monitored for 8 days (Figure

1 [Figure 1: see original paper]). SelX knockdown reduced 570 nm absorbance values starting from day 3, with siSelX showing significantly slower growth than CK from days 3-7. By day 8, no difference was observed between groups.

2.2 Effects of SelX Knockdown and H O Treatment on LO2 Cell Morphology

Morphological changes are shown in Figure 2 [Figure 2: see original paper]. SelX knockdown alone did not affect LO2 cell morphology, with no visible differences between CK and siSelX. However, 200 mol/L H O treatment for 2.5 h induced rounding in both CK and siSelX cells.

2.3 Effects of SelX Knockdown and H O Treatment on Selenoprotein Gene Expression

Gene expression results are presented in Figure 3 [Figure 3: see original paper]. SelX mRNA expression in siSelX cells was significantly lower than CK ($P<0.05$), at only 26% of control levels. SelX knockdown significantly upregulated Gpx6, Txnrd2, and Seli expression ($P<0.05$) while downregulating Dio2, Dio3, Selm, and Sepw1 ($P<0.05$). H O treatment alone significantly upregulated Dio1 ($P<0.05$) and downregulated Gpx2 ($P<0.05$). Combined SelX knockdown and H O treatment significantly upregulated Gpx6, Txnrd2, and Dio1 ($P<0.05$) and downregulated Txnrd3, Dio2, Selm, Sepw1, and Selv ($P<0.05$).

2.4 Effects of SelX Knockdown and H O Treatment on Antioxidant Enzyme Activities

Antioxidant enzyme activities are shown in Table 2 . SelX knockdown significantly increased GSH-Px activity by 66.6% ($P<0.05$) and SOD activity by 51.9% ($P<0.05$), with no effect on T-AOC ($P>0.05$). H O treatment alone did not significantly affect GSH-Px, SOD, or T-AOC ($P>0.05$). Combined SelX knockdown and H O treatment increased GSH-Px activity 1.97-fold compared to CK ($P<0.05$), decreased SOD activity by 19.8% compared to siSelX ($P<0.05$) but not compared to CK ($P>0.05$), and showed no significant effect on T-AOC ($P>0.05$).

Gene silencing is a powerful tool for studying gene function. The low sequence homology among selenoprotein 3' -UTRs enables specific knockdown via RNA interference targeting these regions, making it an important approach for selenoprotein research. Building on our previously established stable SelX-knockdown LO2 cell line, this study further examined how SelX knockdown affects other selenoprotein genes. Selenoproteins are crucial for growth, reproduction, and antioxidant defense. Previous studies show that silencing certain selenoprotein genes reduces cell growth rates—for example, Txnrd1 knockdown decreased TMCK-1 cell growth by ~10%, while Sep15 knockdown significantly reduced HCT116 and HT29 cell growth. Here, SelX knockdown significantly reduced

LO2 cell growth rate on days 3-7 without affecting morphology, while H O treatment caused morphological changes in both CK and siSelX cells.

Twenty-five selenoproteins have been identified in mammals, with many performing vital functions, but potential functional complementation or regulatory interactions remain unclear. This study investigated how SelX knockdown influences other selenoprotein genes. SelX knockdown upregulated Gpx6, Txnrd2, and Seli while downregulating Dio2, Dio3, Selm, and Sepw1. Among upregulated genes, Gpx6 provides antioxidant protection, while Txnrd2, present in liver, kidney, and heart, catalyzes various redox systems using NADPH. Downregulated genes included the deiodinases Dio2 and Dio3, which regulate thyroid hormones and metabolism; SelM, a thiol-disulfide isomerase potentially involved in endoplasmic reticulum disulfide bond formation; and SelW, with antioxidant/redox functions. Genes are regulated through complex networks with potential functional interactions—when one gene is suppressed, others may increase expression to compensate. The altered expression of these selenoproteins following SelX knockdown suggests functional complementation or regulatory relationships requiring further investigation.

The liver is a vital metabolic organ, but whether SelX knockdown affects hepatocyte antioxidant defense was unknown. Excessive ROS production causes oxidative damage, with H O being a major ROS component. Different cell types show varying sensitivity to H O -induced oxidative stress depending on concentration and duration. This study examined combined SelX knockdown and H O challenge effects on selenoprotein expression. The combined treatment upregulated Gpx6, Txnrd2, and Dio1 while downregulating Txnrd3, Dio2, Selm, Sepw1, and Selv. Notably, Gpx6, Txnrd2, Dio1, Dio2, Selm, and Sepw1 showed significant differential expression under either SelX knockdown or H O alone, suggesting potential additive oxidative stress effects. Conversely, Gpx2, Seli, and Dio3 showed significant changes only under single treatments, suggesting possible compensatory effects.

As an important oxidoreductase, SelX protects against oxidative damage. Our previous study found that combined SelX knockdown and H O treatment significantly increased LO2 cell apoptosis. The current study shows that SelX knockdown alone or with H O significantly increased GSH-Px and SOD activities, possibly reflecting a feedback mechanism where reduced redox function from SelX loss triggers increased GSH-Px and SOD secretion to maintain metabolic balance. H O alone did not significantly affect GSH-Px or SOD activities, contrasting with studies showing decreased activities in human umbilical vein endothelial cells, hLE cells, and PC12 cells—likely due to our lower H O concentration and shorter treatment duration.

Conclusions

1. SelX knockdown significantly reduced the growth rate of liver LO2 cells.
2. SelX knockdown and H O treatment affected the expression of other se-

lenoprotein genes in liver LO2 cells.
3. SelX knockdown and H O treatment modulated GSH-Px and SOD activities in liver LO2 cells.

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Note: Figure translations are in progress. See original paper for figures.