4 MATERIALS AND METHODS

4.1 Chemicals

All chemicals were of analytical grade and obtained from SIGMA (München, Germany) or MERCK (Darmstadt, Germany) if not otherwise indicated.

4.2 Cell culture

4.2.1 Cell culture conditions and treatment

Most of the experiments were performed on BV2 cells. BV2 microglial cells are primary mouse microglial cells immortalized by stable transfection with the *c-myc*-oncogene using a J7-retrovirus (93), leading to a phenotype functionally identical to primary microglial cells. BV2 were grown and maintained in Dulbecco´s modified Eagle´s medium (DMEM) (4.5 g/l glucose, 580 mg/ L-glutamine [4 mM], without sodium pyruvate) supplemented with 10 % fetal bovine serum (FBS), 50 U penicillin and 50 µg/ml streptomycin.

The COS-7 cells (African green monkey kidney fibroblast-like cells) (94) were used to test the translation efficiency of Gpx1-EGFP-C1 plasmid by immunoblot and to check the knock-down effect of the siRNA plasmids. COS-7 cells were cultured in DMEM medium like the BV2.

All types of cells were grown in an atmosphere of 5 % CO_2 at a temperature of 37 °C. Proteolytic enzymes such as trypsin in combination with EDTA were used to detach the cells from the growth surface to harvest or to subculture them. Frozen cells were thawed after placing the vials in a 37 °C water bath by gently agitating the vial, the cell suspension was then pipetted into a plate containing the pre-warmed growth medium. For long-term storage cells were harvested as described above and washed once with the complete medium. The cells were then resuspended in the complete medium and counted. They were stored in a medium with 10 % DMSO. Aliquots (about 10^6 cells/ml/cryovial) were frozen and kept in liquid nitrogen.

Activation of BV2 and oxidative stress were induced by addition of 250 µM H_2O_2 (Sigma). Cells were also treated with LPS and IFNγ at the concentration and for the time indicated.

The treatment with selenium was performed by incubation with the indicated concentration of sodium selenite (Na_2SeO_3) for 16 hours (usually overnight).

All experiments were performed using untreated cells as a control, with about 15 nM selenium in the culture medium.

BV2 and COS7 cells were kindly supplied by the Institute of Cell Biology and Neurobiology, Center for Anatomy Charité-University Medical School Berlin (Germany).

4.2.2 Primary microglial cells

Primary microglial cell cultures were prepared from the brains of newborn Wistar rats (postnatal days 1-2) born from rats fed a selenium-deficient (selenium content 5-10 µg/kg) or a selenium sufficient (300 µg Se/kg) diet for several generations. The brains were removed and placed in Dulbecco´s modified Eagle´s medium (DMEM), supplemented with 10 % fetal bovine serum (FBS) and pen/strep. Then the meninges and blood vessels were removed under a microscope. After mechanical dispersion, aliquots of the cell suspension were plated in a poly-D-lysine coated flask. Cultures containing mainly astrocytes and microglial cells were maintained at 37 °C in a humidified 5 % CO_2 atmosphere for 10 days, with the change of the medium every 3-4 days. The cultures were then shaken on an orbital shaker at 150 rpm for 60 min at 37 °C. Floating cells in the supernatant were collected, centrifuged at 250 g for 5 min, and resuspended in the culture medium. This fraction contained the microglial cells which were used for experiments on primary selenium-deficient and selenium sufficient cells.

4.3 Biological and biochemical methods

4.3.1 Test of cell viability (MTT test) (95)

Cell respiration, as an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazon. Cells were seeded in 96-well microtiter dishes (100 µl pro well of 4000-6000 cells/ml) and cultured for 16 hours. On the following day, sodium selenite (Na_2SeO_3) was added and then freshly prepared hydrogen peroxide (H_2O_2). The reaction was started by adding 10 µl/well of a 5 mg/ml MTT stock in Dulbecco´s phosphate-buffered saline (PBS). The cells were incubated for additional 3-4 hours at 37 °C. Thereafter, the medium was removed and the cells were lysed in isopropanol-HCl (100:1). The conversion of MTT into formazon by metabolically viable cells was monitored by a microplate reader at 570 nm. The results were presented as a percentage of the viability of untreated cells (control), which were regarded as 100 % viable.

4.3.2 Propidium iodide staining (measurement of cell death)

Propidium iodide (PI) is a DNA-intercalating dye that is excluded by cells that have their plasma membrane integrity preserved, but enters and stains DNA in cells that have damaged membranes. These are considered dead cells. PI is visible by fluorescence microscopy. This measurement of cell death was applied for primary microglial cells. Cells were seeded in 96-well plates at the density indicated for the MTT test and either incubated with the desired concentration of H_2O_2 or left untreated. Then propidium iodide was added (5 µg/ml, incubated for 5 min), and the cells were observed under a fluorescence microscope to visualize the dead cells (the peak excitation λ and emission λ for propidium iodide is 536 nm and 620 nm, respectively).

4.3.3 Measurement of intracellular ROS (Reactive Oxygen Species) (96)

To detect ROS production, cells were seeded in 96-well plates as described above (MTT test) and then treated with 2.7-dichlorofluorescin diacetate (DCFH-DA) (50 μM) (Molecular Probes Europe BV) at 37 °C, 5 % CO_2. DCFH-DA is a redox-sensitive dye which is readily taken up by the cells. Within the cell, esterases cleave the acetate groups on DCFH-DA, thus trapping the reduced form of the probe (DCFH) intracellularly. ROS in the cells oxidize DCFH, yielding the fluorescent product DCF. After 30 minutes, the fluorescence was measured on a Fluorostar fluorescence plate reader using an excitation wavelength of 488 nm and an emitter band-pass of 510 nm.

4.3.4 Measurement of nitric oxide production (97)

Nitric oxide produced by microglial cells was quantified according to the Griess assay (98) as follows. After BV2 cells had been plated and treated with sodium selenite and activating agents, NO_2^- in culture supernatants was measured to assess the NO production in microglial cells. Then 50 μl aliquots of the sample were mixed with 50 μl of Griess' reagent for nitrite (1 % sulfanilamide/0.1 % naphthylethylene diamine dihydrochloride/2 % phosphoric acid) in a 96-well plate and incubated at 25 °C for 10 min. The absorbance at 570 nm was measured by a microplate reader. Sodium nitrite ($NaNO_2$) was used as the standard to calculate the NO_2^- concentrations.

4.3.5 Total protein preparation (99)

For total protein extraction, control cells and treated cells were collected, washed with ice-cold PBS and lysed in a low volume of freshly made lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 % TritonX-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 % sodium dodecyl sulphate (SDS), protease inhibitor cocktail (1 tablet in 25 ml buffer) (Boehringer Complete, Roche, Mannheim, Germany). The homogenate was sonicated for 5 minutes at 4 °C to disrupt

the protein-DNA interaction. After incubation on ice for 15 min, the lysate was clarified by centrifugation at 15000 x g for 10 min at 4 °C. The supernatant (soluble fraction) was aliquoted and shock-frozen in liquid nitrogen. An aliquot was prepared for the protein detection.

4.3.6 Preparation of cytoplasmic and nuclear extracts (100)

To investigate the effect of selenite on NF-kappa B and iNOS activation, Western blots were performed on nuclear and cytosolic extracts, respectively. Adherent cells were scratched with a cell scraper in cold PBS. The cell suspension was pelleted by centrifugation and resuspended in PBS. All subsequent steps were performed at 4 °C. The cells were incubated for 17 min in 400 µl of a hypoosmotic buffer (10 mM Hepes, pH 7.8, 10 mM KC1, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 µg/ml aprotinin, leupeptin 0.5 µg/ml, 3 mM PMSF, and 3 mM DTT). 25 µl of 10 % NP-40 was added and the nuclei were pelleted by centrifugation for 5 min in a microfuge. The supernatants containing the cytoplasmic proteins were removed and stored at -70 °C. The pelleted nuclei were resuspended in a salt buffer (50 mM Hepes, pH 7.4, 50 mM KCI, 300 mM NaC1, 0.1 mM EDTA, 10 % (vol/vol) glycerol, 3 mM DTT, and 3 mM PMSF) to solubilize DNA-binding proteins. The resuspended nuclei were shaken gently for 30 min at 4 °C. The extracts were spun in a microcentrifuge for 10 min and the clear supernatants containing nuclear proteins were aliquoted and stored at -70 °C.

4.3.7 Protein quantification: BCA™ assay (101)

The protein concentration of the extracts was determined according to the standard protocol of "Bicinchoninic Acid (BCA™) Protein Assay Kit" from Pierce (UK). A bovine serum albumin (BSA) standard was used.

4.3.8 One-dimensional polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis is a bioanalytical tool used in fundamental research for the isolation and identification of proteins. The separation is based on differences in the mobility of charged macromolecules under the influence of an electric field. Polyacrylamide gel electrophoresis (PAGE) was used for the separation of proteins. Polyacrylamide gels are prepared by the reaction of acrylamide (monomer) with N,N´-methylenebis(acrylamide) (cross linker) in the presence of a catalyst (TEMED, tetrametylethylenediamine) and an initiator (APS, ammonium persulfate).
The protein samples were separated in 12 % resolving gels and 3.75 % stacking gels according to the standard protocol of Laemmli (102). The samples were diluted in SDS-sample buffer (4 % SDS, 12.5 mM Tris-HCl pH 8.8, 20 % glycerol, 20 mM DTT, 0.2 % bromophenol blue) and heated for 5 min at 95 °C in a "float" in a waterbath. The final tank buffer containing 196 mM glycine / 0.1 % SDS / 50 mM Tris-HCl pH 8.3 was put into both top and bottom tanks. The separation was performed at 200 V until the bromophenol blue front had left the gel. The molecular mass was determined taking the Biolabs prestained Protein Marker (broad range) as a marker.

4.3.9 Western blot analysis

4.3.9.1 Blotting and staining of proteins on membranes

Western blotting or immunoblotting allows to determine, with a specific primary antibody, the relative amounts of the protein present in the samples. Samples of protein mixtures (cell lysates, nuclear or cytosolic fractions) were electrophoresed as described above and then transferred onto nitrocellulose membranes (0.45 µm pore size) (Sigma) using the semi-dry transfer system (Trans-Blot SD, Bio-Rad). The transfer was carried out for 50 min at 20 Volts, using a blotting buffer containing 192 mM glycine, 25 mM Tris and 20 % methanol. The membrane was washed with PBS and stained with 0.2 % Ponceau S (Sigma) for checking the successful transfer, then destained in PBS for several minutes on a shaker.

4.3.9.2 Blocking and antibody incubation

Nitrocellulose blots were soaked overnight at 4 °C in blocking buffer (5 % non-fat milk powder in PBS) to saturate all non-specific protein binding sites on the blots. The blots were then washed 3 times for 10 min with PBS and incubated with the first antibody for 2 hours at room temperature or overnight at 4 °C. After extensive washing with PBS, the blots were incubated with a HRP (Horseradish Peroxidase)-conjugated secondary antibody. Primary and secondary antibodies were used at the following dilutions:

Anti-NF-kß P65 (Sigma)	1:5000
anti-iNOS/NOS Type II (BD biosciences)	1:2500
Anti-Caspase-3 (Biosource, Belgium)	1:1000
Anti-TrxR1 (Prof. Dr. K.Becker-Brandenburg, Gießen)	1:5000
Anti-SelP (Dr. U. Schweizer, Berlin)	1:5000
Anti-ß-Actin (Sigma)	1:5000
Anti-Aequorea victoria GFP (JL-8) (Clontech)	1:2500
ECL Anti-rabbit IgG, peroxidase-linked (Amersham)	1:5000
ECL Anti-mouse IgG, peroxidase-linked (Amersham)	1:5000

4.3.9.3 Detection

The protein-antibody complexes were visualized by chemiluminescence using the ECL™ Western blotting detection reagents from Amersham Pharmacia Biotech. ECL films were scanned with PowerScan U1120 (Biostep), assembled and annotated using Adobe Photoshop. Densitometry was used to quantify the signals from the Western blot analysis (program Metamorph - Universal Imaging, Downingtown, PA).

4.3.9.4 Stripping and ß-actin immunoassay

To check another antibody, the membrane was stripped by soaking in 100 mM sodium citrate pH 2.2 for 15 min. After washing with PBS and blocking, the blots were reprobed with a primary antibody and the procedure of incubation was carried out as described previously. Controls for equal loading of the blots were achieved

with immunoanalysis for the anti-mouse ß-actin monoclonal antibody (Sigma) after stripping of the membrane.

4.3.10 2D PAGE (103)

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is a widely used method for separating a large number of proteins from complex protein mixtures and for revealing patterns of protein expression.
The system Multiphor™ II Electrophoresis unit with Immobiline™ (Amersham) was used, and the protocol from the user manual was followed.
The first dimension of the 2D PAGE technique resolves proteins by isoelectric focusing (IEF). All proteins have a net charge (the sum of the charges of the amino acid side chains). The pH at which the net charge of the protein equals zero is the isoelectric point (pI). IEF separates (focuses) proteins on the basis of their charge or pI by electrophoresis in a polyacrylamide gel in a pH gradient. After completion of IEF, the proteins are resolved in the second dimension via SDS-PAGE. After equilibration of the IPG strips they are affixed to the top of a vertical SDS-polyacrylamide gel and electrophoresed. Finally, the separated proteins are visualized. Proteins labelled with ^{75}Se were detected by autoradiography; another method of detection used for two-dimensional gels was silver staining.

4.3.11 Staining of protein gels

The most common visualization method involves staining an entire gel with a species that interacts in a non-selective way with proteins. Two different staining methods were used.

4.3.11.1 Staining with Coomassie Brilliant Blue R-250

Protein gels were removed from the electrophoresis apparatus and stained for at least 15 min in a Coomassie staining solution (30 % (v/v) ethanol, 10 % (v/v) acetic acid, 0.2 % (w/v) Coomassie Brilliant Blue R-250). The staining solution was removed,

and the gels were immersed in 30 % (v/v) ethanol, 10 % (v/v) acetic acid, 60 % (v/v) water. After destaining, the gels were fixed by immersion in 10 % (v/v) glycerol, 7 % (v/v) acetic acid and 5 % (v/v) ethanol for about 3 hours.

4.3.11.2 Staining with silver nitrate (according to Rabilloud T.) (104)

After fixation for 3 hours in 30 % (v/v) ethanol, 10 % (v/v) acetic acid, 60 % (v/v) water, the gels were washed three times with water for 10 minutes and incubated for 1 min in 0.02 % (w/v) sodium thiosulfate. After washing twice with water, the gels were incubated in a silver nitrate solution (0.2 % (w/v) silver nitrate, 0.03 % (v/v) formaldehyde) in the dark for 1 h. After washing for 30 sec, the gels were developed for at least 3 min in 3 % (w/v) sodium carbonate, 12 mM sodium thiosulfate and 0.03 % (v/v) formaldehyde. The staining process was stopped by applying acetic acid. The gels were then fixed by immersion in 10 % (v/v) glycerol, 7 % (v/v) acetic acid and 5 % (v/v) ethanol for about 3 hours.

4.3.12 Drying of gels in a vacuum

Gels were placed into a destaining solution containing glycerol before drying. This reduces cracking by minimizing the shrinkage that normally occurs during this process. The gels were dried between cellophane membranes using a gel drier (Biometra D62) under vacuum for about 20 h at 70 °C.

4.4 Molecular biological methods

4.4.1 Cloning of the Gpx1-EGFP-C1 vector

4.4.1.1 Overview

PCR with two specific primers for the DNA amplification
Agarose gel electrophoresis and extraction of DNA from gel
Ligation of the PCR product into a pCR®2.1-TOPO® vector
Transformation in *E. Coli*, Colony Screening
Mini DNA-preparation, restriction digestion
Agarose Gel and Geleluition of the construct
Ligation of the construct into the pEGFP-C1 vector
Transformation in *E. Coli*, Colony Screening
Mini DNA-preparation, restriction digestion
Maxi DNA-preparation, restriction digestion
Sequencing and Blast

The cloning process was started by DNA amplification (PCR) of the Gpx1 full length DNA using two specifically designed primers (up- and down-stream targeted sequence). The PCR product was checked on agarose gel, eluted and ligated into the pCR®2.1-TOPO® vector. Competent *E. Coli* cells were transformed and the vector was isolated. After cleavage of the full length construct, the insert was ligated into the pEGFP-C1 vector. Competent *E. Coli* cells were again transformed, the vector was isolated and sequenced for cell transfection experiments.

4.4.1.2 Database search and primer design

The Gpx1 nucleotide sequence was obtained from the GenBank database. The GenBank sequence database at the National Center of Biotechnology Information was accessed via the internet world wide web (http://www.ncbi.nlm.nih.gov/).
The putative SECIS element sequence was searched with the SECISearch program on the website http://genome.unl.edu/SECISearch.html (39).

For forward and reverse primer design, the software DNA calculator (Sigma GenoSys, https://www.sigma-genosys.com/order_DNACalc.asp) was used.

4.4.1.3 PCR (Polymerase Chain Reaction)

The purpose of a PCR is to make a huge number of copies of a gene. PCR uses a thermostable DNA polymerase and two short oligonucleotide DNA sequences (primers). Each primer is complementary in sequence to a short length of one of the two strands of DNA to be amplified.

The DNA duplex was heated to 94 °C to separate the two strands. The mixture was then cooled to 61 °C to allow the primers to anneal to their complementary sequences. At 72 °C the primers directed the DNA polymerase to copy each of the template strands.

The process of template denaturation, primer annealing and DNA synthesis was repeated many times to yield many thousands of copies of the original target sequence.

The PCR reaction volumes were:

5µl 10 x Titanium Taq-PCR Buffer
1µl 50 x dNTPs Mix
1µl 10µM forward primer
1µl 10µM reverse primer
1µl Gpx1-pcDNA (cDNA)
1µl 50 x Titanium Taq DNA polymerase
ad 50 µl *a. dest.*

4.4.1.4 Agarose gel electrophoresis of DNA

The amplified cDNA was separated in an 1.5 % (w/v) agarose gel. To pour a gel, agarose powder was mixed with electrophoresis buffer (TAE) to the desired concentration, then heated in a microwave oven until completely molten. Ethidium bromide (final concentration 0.5 µg/ml) was added to the gel at this point to facilitate the visualization of the DNA after electrophoresis. After cooling the solution to about

60 °C, it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature or in a refrigerator. After the gel had solidified and the comb had been removed, it was inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading buffer were then pipetted into the sample wells. The lid and power leads were placed on the apparatus, and a current was applied (100 V).

When adequate migration had occurred, the gel was placed on a ultraviolet transilluminator and the DNA fragments were visualized by staining with ethidium bromide (EtBr, a molecule which binds to DNA and fluoresces when excited by ultraviolet light).

4.4.1.5 DNA extraction from the agarose gel

The resulting DNA fragment was then excised from the agarose gel with a clean scalpel and purified according to the standard protocol of NucleoSpin® Extract II kit (MACHEREY-NAGEL). The eluted DNA was stored at -20 °C.

4.4.1.6 TOPO® cloning reaction (105)

The purified Taq polymerase-amplified PCR product was cloned into a pCR®2.1-TOPO® using TOPO®TA Cloning Kit (Invitrogen). The TOPO® cloning reaction was set up as follows for the transformation into chemically competent *E. Coli*:

REAGENT	VOLUME
PCR product	4µl
Salt Solution	1µl
TOPO® vector	1µl
Final Volume	6µl

The mixture was gently mixed and incubated for 10 minutes at room temperature. It was then placed on ice and the One Shot® Chemical Transformation was carried out.

4.4.1.7 Transformation in competent *E. Coli* (One Shot® Chemical Transformation)

3 µl of the TOPO® Cloning solution were added on the top of a vial of One Shot® Chemically Competent *E .Coli* (Genotype TOP10F´), mixed gently and incubated for 20 minutes on ice. The transformation was stopped by heating the cells for 30 seconds at 42 °C on a water bath without shaking. The tube was then transferred to ice and 700 µl of the SOC medium (room temperature) were added. The tube was shaken horizontally (450 rpm) at 37 °C for 1 hour.
100 µl from each transformation were spread on a pre-warmed agar plate containing an antibiotic and pre-treated with X-gal (40 µl of 40 mg/ml) and IPTG (40 µl of 100mM). For TOP10F´ cells, IPTG was required for blue/white screening. The plates were then inverted and placed overnight at 37 °C (12-16 hours).

4.4.1.7.1 Medium and agar plates for bacteria

SOC medium	2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
2X YT medium	16 g Bacto-tryptone, 10 g Bacto-yeast extract, 5 g NaCl ad 1000 ml deionized water, pH 7.0.
2X YT agar plates	1.5 g/L Agar was added to the 2X YT medium and autoclaved for 20 min at 120 °C. After autoclaving, the mixture was cooled to ~ 50 °C and the antibiotic was added (100 µg/ml). The resulting agar medium was poured into 10 cm plates and stored inverted at 4 °C.

4.4.1.8 Colony screening

The pCR®2.1-TOPO® vector was manufactured commercially. It included some form of signal that indicates when DNA has been inserted into the plasmid's ring of DNA. It was engineered to contain a multiple cloning site (MCS) which has a lac-Z gene attached. When inserted into a suitable bacterial cell, a functional MCS (Lac-Z gene) will produce beta-glactosidase which produces blue colonies. Therefore, if DNA (the fragment being incorporated) ligates successfully into the MCS, the lac-Z gene is disrupted, and the host bacteria appear as white colonies (positives). White colonies were screened by looking for the best white colony representatives (these being those that had further satellite colonies around them). Single positive colonies were picked using sterile toothpicks. The toothpick was inserted gently into the chosen colony and transferred to a 5 ml aliquot of the 2X YT medium containing 100 µg antibiotic /ml. The culture was shaken (180 rpm) overnight (12-16 hours) at 37 °C.

After incubation, 1 ml of the solution was mixed with 1 ml of the 2X YT medium containing glycerol, vortexed and then stored at -80 °C (this snap-frozen sample can be stored for long periods).

4.4.1.9 Plasmid DNA mini-purification

For the quick purification of the plasmid DNA, the SEQLAB Mini-Prep kit was used. 2-5 ml of the overnight culture were centrifuged for 10 min at 4000 rpm. The supernatant was completely removed and the pellet resupended in 100 µl resuspension buffer RE containing RNase. 200 µl lysis buffer LY were added, the tube was moved gently overhead and incubated for maximal 5 min at room temperature. Then 150 µl neutralisation buffer NE were added, the tube was moved gently overhead and incubated for about 5 min at room temperature. The tube was centrifuged at ≥ 10000 x g for 30 min and the supernatant was transferred to a new 1.5 ml Eppendorf tube. 2 vol. of pure ethanol were added, the solution was mixed well and centrifuged at ≥ 10000 x g for 20 min. The pellet thus formed was washed with 1 ml 70 % ethanol and incubated for 5 min at room temperature. The tube was centrifuged for another 5 min, the resulting pellet was dried and redissolved in 30 µl of pure water. The purified DNA plasmid was stored at -20 °C.

4.4.1.10 Restriction digestion

After the plasmid DNA mini-purification, the plasmids were analyzed by restriction digestion to confirm the presence of the insert. Restriction digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called restriction enzymes (RE's). These special enzymes recognize specific sequences in the DNA molecule wherever that sequence occurs in the DNA. A combination of two restriction enzymes that cut once in the construct was used. The Gpx1 insert was cut from the vector with *Hind III* and *Bam HI*.

The reagents used in the restriction digestion for Gpx1-pCR®2.1-TOPO® were:

1 µl DNA from mini-prep (~ 1µg/µl)
1 µl *Hind III* (20 U/µl)
1 µl *Bam HI* (20 U/µl)
0.5 µl BSA (10 mg/ml)
5 µl buffer *Bam HI* (10X)
ad 50 µl *a. dest.*

Once all the ingredients were mixed in the reaction tube, the tube was incubated at 37 °C for 1-2 hours. After the restriction digest had been completed, agarose gel electrophoresis was performed to separate the digest fragments by size and visualize the fragments.

4.4.1.11 Maxi restriction digestion of vector and insert

To produce a large amount of insert and empty vector necessary for the following ligation, a maxi restriction digestion with the two restriction enzymes was carried out. The digestion produced cohesive ends. The Gpx1 insert was obtained from a positive mini-purification, and the empty vector was obtained from cutting the pEGFP-C1 vector.

5 µl DNA
1 µl *Hind III* (20 U/µl)
1 µl *Bam HI* (20 U/µl)
0.5 µl BSA (10 mg/ml)
5 µl buffer *Bam HI* (10X)
ad 50 µl *a. dest.*

The mix was incubated overnight at 37 °C to allow the complete digestion of DNA.

4.4.1.12 DNA gel electrophoresis, gel elution and determination of the DNA concentration

After the overnight restriction digestion, agarose gel electrophoresis was performed and the DNA fragments (insert and vector) were excised and purified according to the standard protocol of the NucleoSpin® Extract II kit (MACHEREY-NAGEL). The DNA samples were run on an agarose gel alongside known amounts of DNA of a similar size. The amount of sample DNA loaded can be estimated by visual comparison of the band intensity with the standards.
The intensity of the fluorescence is related to the amount of EtBr bound, and this is depended on the number of base pairs.

4.4.1.13 Dephosphorylation of the vector prior to cloning

The restriction-digested pEGFP-C1 vector was dephosphorylated using SAP (Alkaline Phosphatase, shrimp). The removal of the 5'-terminal phosphates from the dsDNA ends prevents vector self-ligation and improves the ligation results.

1 µl DNA
0.5 µl SAP (10 u/ml)
0.5 µl SAP buffer (10X)
ad 5 µl *a. dest.*

The mix reaction was incubated for 30 minutes at 37 °C and was stopped by heating at 65 °C for 30 minutes. This completely inactivates SAP.

4.4.1.14 Ligation into pEGFP-C1 and transformation

Ligation is the process by which a DNA fragment (insert) is inserted into an appropriate plasmid (vector). The Gpx1 insert was ligated into the pEGFP-C1 vector using the enzyme T4 DNA ligase. This enzyme ligates DNA fragments having overhanging, cohesive ends that are annealed together.
The concentration of the vector was set up at 200 ng and the concentration of the insert was calculated using the following formula:

$$\frac{\text{vector (200 ng)} \times \text{insert (kb)}}{\text{vector (kb)}} \times \frac{\text{ratio of insert}}{\text{vector}} = \text{insert (? ng)}$$

A 3:1 (insert:vector) ratio was used for the ligation reaction. The DNA ligation procedure was carried out as follows:
The components of the NE-Biolabs ligase kit were added together, vortexed and placed overnight at 16 °C. The same volumes were used for both positive and negative (without insert) control reactions (adjusting volumes with *aqua dest.*).

200 ng pEGFP-C1 vector
115 ng Gpx1 insert
1 µl ligase buffer (10X)
1 µl T4 DNA ligase
ad 10 µl *a. dest.*

The ligation reaction was transformed in competent E. Coli as described in 4.4.1.7 and the transformed competent cell cultures were aliquoted to ampicillin/agar-plates (freshly prepared). The plates were inverted and placed overnight at 37 °C. The following day the samples were removed form the oven, placed at 4 °C and screened for positive colonies.

4.4.1.15 Colony screening and plasmid purification

About 8 colonies were screened for positive clones as described previously (paragraphs 4.4.1.8 to 4.4.1.10). One positive colony was picked and cultured in 200 ml of 2X YT medium containing 100 µg antibiotic /ml. The culture was shaken (180 rpm) overnight (12-16 hours) at 37 °C. The day after, the isolation of the DNA was performed using the NucleoBond Plasmid Maxi EF (endotoxinfree) kit. The protocol used is summarized as follows. The overnight bacterial culture was centrifuged at 6000 x g for 15 min at 4 °C. The supernatant was carefully discarded and the pellet resuspended in 12 ml buffer S1-EF + RNase A. 12 ml of buffer S2-EF were then added to the suspension and mixed 6 times by inverting the tube. The mixture was incubated at room temperature for about 3 min. After that, 12 ml of buffer S3-EF were added and mixed 6 times by inverting the tube. A heavy flocculent precipitate was formed. The lysate was cleared first by centrifugation at ≥ 12000 x g for 30 min and then by filtering the suspension with a folded filter.

A NucleoBond AX 500 EF column was equilibrated with 5 ml of buffer B2-EF and the cleared lysate was loaded onto the column. After washing twice with buffer N3-EF (24 ml) and twice with buffer N4-EF (12 ml), the plasmid DNA was eluted with 15 ml of buffer N5-EF. To precipitate the eluted DNA, 0.7-0.8 volumes isopropanol were added and then centrifugated at > 10000 x g for 30 min at 4 °C.

The pellet thus formed was redissolved in 30 µl water and was subjected to measurement of purity and yield as described below.

4.4.1.16 Determination of the concentration and purity of DNA

The concentration of DNA was determined by spectrophotometric estimation. The concentration of pure double-stranded DNA with an A_{260} of 1.0 is 50 µg/ml. Thus, the following formula was used to determine the DNA concentration of a solution.

Unknown (µg/ml) = 50 µg/ml x measured A_{260} x dilution factor

The assessment of the purity of the DNA was performed by calculating the A_{260}/A_{280} ratio. Pure DNA has A_{260}/A_{280} ratios of ≥1.8, based on the extinction coefficients of

nucleic acids at 260 nm and 280 nm. Smaller ratios usually indicate contamination by protein or organic chemicals.

4.4.1.17 Sequencing and blast

Plasmids were sequenced to verify that they contained the desired inserts and to control for unwanted mutations. The samples were sequenced by the company MWG Biotech AG, Ebersberg (Germany). The obtained sequences were compared with the Gpx1 sequence using the "BLAST 2 SEQUENCES" tool (106).

4.4.2 Cloning of siRNA vectors

4.4.2.1 Overview

The pSUPER RNAi system (www.oligoengine.com) was used for efficient and specific down-regulation of Gpx1 gene expression, resulting in functional inactivation of the targeted genes. The expression of siRNAs using this vector mediates a transient suppression of gene expression, allowing the analysis of loss-of-function phenotypes (107).

Steps

Oligo insert design
Annealing and phosphorylation of oligos
Ligation into pSUPER-GFP vector
Transformation in *E. Coli*, Colony Screening
Mini DNA-preparation, restriction digestion
Maxi DNA-preparation, restriction digestion
Sequencing and blast

4.4.2.2 Oligo insert design

To achieve the silencing of a Gpx1 gene, the pSUPER-GFP vector was used in concert with a pair of custom oligonucleotides that contain a unique 19-nt sequence derived from the mRNA transcript of the Gpx1 gene (the "N-19 target sequence").
The N-19 target sequence corresponds to the sense strand of the pSUPER-generated siRNA, which in turn corresponds to a 19-nt sequence within the mRNA. In the mechanism of RNAi, the antisense strand of the siRNA duplex hybridizes to this region of the mRNA to mediate the cleavage of the molecule.
For target selection a design tool on the webpage http://www.ic.sunysb.edu/stu/shilin/rnai.html was used. The tool generated N-19 target sequences for the Gpx1 gene.

Three 19-nt target sequences were selected according to the 8 criteria found by A. Reynolds et al. (108) for functional siRNA:

1. 30 % - 52 % ´G/C´ content
2. At least 3 ´A/U´ bases at position 15-19 (sense strand)
3. Absence of internal repeats
4. An ´A´ base at position 19 (sense strand)
5. An ´A´ base at position 3 (sense strand)
6. A ´U´ base at position 10 (sense strand)
7. A base other than ´G´ or ´C´ at 19 (sense strand)
8. A base other than ´G´ at position 13 (sense strand)

The selected 19-nt target sequences are in the coding region, 100 bp down from the start and 100 bp up from the termination of translation to avoid interference by RNA regulatory proteins that bind the 5' or 3' UTR.

The sequence of the forward oligo includes the unique N-19 target in both sense and antisense orientation, separated by a 9-nt spacer sequence. The 5' end corresponds to the *BglII* site, while the 3' end contains the T5 sequence and any *HindIII* corresponding nucleotides.

Forward oligo model:

5` - GATCCCC **Target sequence: sense** TTCAAGAGA **Target sequence: antisense** TTTTTGGAAA - 3´

Reverse oligo model:

5´ - AGCTTTTCCAAAAA **Target sequence: sense** TCTCTTGAA **Target sequence: antisense** GGG - 3´

The 64-nt oligos were obtained from Metabion GmbH (Martinsried, Germany).

These forward and reverse oligos were annealed and cloned into the vector, between the unique *BglII* and *HindIII* enzyme sites. This positions the forward oligo at the correct position downstream from the H1 promoter's TATA box to generate the desired siRNA duplex.

The resulting transcript of the recombinant vector is predicted to fold back on itself to form a 19–base pair stem-loop structure.

The analysis indicates that the stem-loop precursor transcript is quickly cleaved in the cell to produce a functional siRNA.

4.4.2.3 Annealing and phosphorylation of oligos

4.4.2.3.1 Annealing reaction

The forward and reverse strands of the oligos that contain the siRNA-expressing sequence targeting Gpx1 were annealed after being dissolved in sterile water at a concentration of 900 µM. The annealing reaction was assembled by mixing 1 µl of each oligo (forward + reverse) with 48 µl annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM Mg acetate). The mixture was incubated at 90 °C for 4 min, and then at 70 °C for 10 min. For slowly cooling down to 4 °C, a PCR block was used. The annealed oligo inserts were stored at -20 °C or phosphorylated immediately.

4.4.2.3.2 Phosphorylation

2 µl of the annealed oligos
1 µl T4 PNK (Polynucleotide Kinase) buffer
1 µl ATP 1 mM
1 µl T4 PNK
5 µl *a. dest.*

The mixture was incubated for 30 min at 37 °C, and then 10 min at 70 °C (heat inactivation of T4 PNK).

4.4.2.4 Ligation into the pSUPER-GFP vector

The pSUPER-GFP vector was linearized by *BglII/HindIII* and dephosphorylated by SAP (Shrimp Alkaline Phosphatase) treatment. The removal of the 5´-terminal phosphates from the dsDNA ends prevents vector self-ligation and improves the ligation results.

The ligation is the process of joining linear DNA fragments together with covalent bonds.

Ligation reaction:

2 µl of the annealed phosphorylated oligos
1 µl ligase buffer
1 µl pSUPER-GFP vector (digested with *BglII/HindIII* and SAP treated)
1µl T4 DNA ligase
5 µl *a. dest.*

The mixture was incubated at 16 °C overnight and the day after the transformation in *E. Coli* was carried out as described in 4.4.1.7. After the colony screening and Mini- and Maxi-DNA preparations, the constructs were sequenced and finally transfected into the cells.

4.4.3 Transient transfection of cells

Transfections with the generated plasmids were performed using the Amaxa Nucleofector electroporation or the calcium phosphate technique.

4.4.3.1 Amaxa Nucleofector electroporation method

The Nucleofector technology (Amaxa Biosystems, Köln, Germany) is a highly efficient non-viral method for transfection. It is based on two components: the Nucleofector device that delivers unique electrical parameters, and the Nucleofector kits that contain cell-type-specific Nucleofector solutions. This technology achieves an efficient direct transport of the DNA into the cell nucleus. Consequently the expression of the transfected protein can be detected shortly after nucleofection. For transfection of BV2 and COS-7 cells, the Nucleofector™ Kit V was used, by following the manual instructions. BV2 cells were transfected with the A-23 program on the Nucleofector device, COS-7 with the A-24 program.

4.4.3.2 Calcium phosphate transfection

The transient transfection of COS-7 cells for testing the translation efficiency of Gpx1 by immunoblot and checking the knock-down effect of the siRNA plasmids was performed by the calcium phosphate method. The following protocol was adopted from Sambrook and Maniatis (109). This transfection method is commonly used for the transfer to and expression of genetic information. It is a simple and low cost method to transfect efficiently mammalian cells. The principle is that DNA is mixed to a $CaCl_2$-solution before adding a phosphate buffer, which initiates the formation of a calcium-phosphate precipitate. The precipitate is then added to the culture medium and adsorbed at the cell surface through phagocytosis.

24 hours prior transfection, subconfluent COS-7 cells were split in order to get a 50-60 % confluent plate (10 cm petri dish) the next day. One hour before transfection, the medium was replaced by 10 ml of fresh culture medium. The plasmids were prepared in the presence of calcium (250 mM) in 500 µl of the medium. The same amount of 2x HEPES-buffered saline was prepared. The plasmid and calcium mix were added slowly to the 2X HEPES-buffered saline buffer, and the resulting mixture was incubated for 30 min at room temperature. The mixture was vortexed gently and added drop-wise to the culture medium. 48 hours after transfection, the cells were washed twice with PBS and pelleted for subsequent cell extract preparation and Western blot analysis.

4.5 Radiolabelling with ^{75}Se

Profiles of the selenoproteins expressed by the cells were determined by radiolabelling with ^{75}Se-selenite.

The gamma emitter ^{75}Se is commercially available in the form of [Se^{75}]-selenite, with a high specific radioactivity, from the Research Reactor Center, University of Missouri-Columbia, USA. The ^{75}Se isotope has a half-life of 120 days. After incorporation it can easily be analysed by autoradiography.

Confluent cultures of cells in 75 cm^2 flasks were labelled with 2 kBq/ml [Se^{75}]-selenite (specific activity ~ $3.7*10^7$ Bq/g) by incubation for 48 h. The medium was

then decanted and the cells washed twice with PBS before being harvested into PBS by scraping, followed by centrifugation at 300 g for 10 min.
The labelled Se-containing proteins were investigated in cell lysates after separation by SDS-PAGE and autoradiographic determination of the tracer distribution in the gels. ^{75}Se in the samples was measured by means of a 3 x 3" NaJ (Tl) well-type detector coupled to a multi channel analyzer.

4.5.1 Detection of labelled selenoproteins

The tracer distribution in the electrophoretic gels was determined autoradiographically by using a photostimulable phosphor plate which in connection with an imaging analyzer allows the quantitative analysis of the ^{75}Se activity in the separated labelled proteins.
The Fuji BAS1000 system with the *AIDA* software from Raytest (Straubenhardt, Germany) was used. The imaging plate is a highly advanced photosensitive plate composed of a photostimulable phosphor sandwiched between a flexible plastic support and a thin protective layer. The emitted light is amplified and converted into electrical signals by a photomultiplier, resulting in a pixel map with the intensity expressed as photostimulable luminescence units (PSL).

4.6 Fluorescence Activated Cell Sorting (FACS) analysis

4.6.1 Apoptosis (Annexin V staining)

Apoptosis can be defined as "gene-directed cellular self-destruction" or programmed cell death. There are many ways of detecting apoptosis by flow cytometry. Apoptotic cells can be recognised by a characteristic pattern of morphological, biochemical and molecular changes. The method used for detecting apoptosis by flow cytometry was Annexin V staining. One of the membrane changes in the early / intermediate stages of apoptosis is the translocation of phosphatidylserine (PS) from the inner side of the cell membrane to the outside. It is possible to detect PS by using FITC-labelled Annexin V, which is a Ca^{++}-dependent phospholipid-binding protein. Apoptotic cells

show positive results for Annexin-FITC. The advantage of this method is that it allows the use of a benchtop analytical flow cytometer.

Apoptosis was detected using the fluorescein-conjugated Annexin V (FITC) antibody from BD Bioscience (Heidelberg, Germany). Cells ($1*10^6$ cells/dish) were treated with sodium selenite (Na_2SeO_3 500 nM) for 16 hours and then exposed to 250 µM H_2O_2 for 4 hours. To prepare the cell sample for flow cytometry, the cells were washed with cold PBS, resuspended in Annexin-binding buffer (ABB) at the concentration of $2.5*10^5$ cells / 100 µl and stained with 5 µl of Annexin V-fluorescein isothiocyanate (FITC) for 15 min at room temperature in the dark. In the negative control, cells were added to the ABB buffer without antibody. 400 µl of ABB were added to each tube and the cells were centrifuged at 300 x g. Each sample was resuspended in 300 µl of ABB and finally analyzed by a Becton Dickinson FACS Flow Cytometer using the FL1 range for Annexin V FITC.

The following controls were used to set up compensation and quadrants: unstained cells and untreated cells stained with Annexin V-FITC.

4.6.2 Adhesion molecules (CD11a)

The surface expression of CD11a on BV2 cells was determined using flow cytometry. After treatment, cells were harvested and fixed with 4 % PFA. Aliquots of $5x10^5$ cells were resuspended with 100 µl FACS buffer. The tubes were centrifuged at 300 x g for 5 minutes. The cell pellets were resuspended in 50 µl FACS buffer containing PE-conjugated monoclonal rat anti-mouse-CD11a antibody (BD Bioscience, dilution 1:100). After 10 minutes of incubation at 4 °C in the dark, 1 ml FACS buffer was added to each sample and centrifuged at 300 x g for 5 minutes. The pellets obtained were resuspended in 300 µl of FACS buffer and finally analyzed by a Becton Dickinson FACS Flow Cytometer using the FL2 range for PE-CD11a.

4.7 Determination of enzyme (Gpx1) activity

The activity of glutathione peroxidase was determined according to the assay described by Paglia and Valentine (110) using the "Glutathione Peroxidase Cellular Activity Assay Kit" from Sigma. The kit uses an indirect determination method. It is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by Gpx, which it is coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH (ß-Nicotinamide Adenine Dinucleotide Phosphate, reduced). The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ is indicative of the Gpx activity, since Gpx is the rate-limiting factor of the coupled reactions.

$$R\text{-}OOH + 2\ GSH \xrightarrow{Gpx} R\text{-}OH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\ GSH + NADP^+$$

The activity of Gpx1 was expressed as % of the control (untreated cells).

4.8 Immuno-histochemistry

For the subcellular localization of the enzyme, COS-7 cells were transfected with Gpx1-EGFP-C1 plasmid using the Amaxa Nucleofector electroporation system and plated on coverslips in a 12-well plate. 24 hours after transfection, the cells were fixed with 4 % paraformaldehyde for 10 min, washed with PBS and permeabilized with 0.5 % Triton X-100 for 5 min. After washing, the cells were blocked with 5 % non-fat milk powder and 0.2 % Tween 20 in PBS for 1 hour at 37 °C. The cells were incubated for 1 hour with the primary antibody diluted in PBS, washed three times and incubated with the secondary antibody (Alexa Fluor 568 goat anti–mouse IgG antibody, dilution 1:800) for 1 h. After three washing steps with PBS, the coverslips were mounted on glass slides with Immu-Mount (Thermo). The mounted cells were analyzed by fluorescence microscopy.

	Monoclonal antibody to	Dilution
Mitochondrial Marker	Prohibitin	1:100
Golgi Marker	58K Golgi protein	1:100
Early Endosome Marker	EEA1	1:500
Late Endosome Marker	Mannose 6-Phosphate Receptor	1:200
Endoplasmic Reticulum (ER)	Calnexin	1:200

All the antibodies were purchased from Acris Antibodies (Hiddenhausen, Germany)

For nuclei colocalization studies, transfected cells were stained with Hoechst 33342 according to the protocol by Bräuer et al (111). The Hoechst 33342 dye is widely used for staining the nuclei of living cells. Hoechst dyes preferentially bind to adenine-thymine (A-T) regions, making them quite selective for DNA; Hoechst dye-stained cells and tissues show virtually no cytoplasmic staining.

4.9 Microscopy

Microscopic cell images were taken with a Color View II Camera (Soft Imaging System) on a Zeiss (Axiovert 25) inverted microscope.
Immunofluorescence analysis was conducted by confocal laser scanning with a Leica TCS SL and an Olympus BX51 upright microscope.

4.10 Determination of the selenium content by atomic absorption spectrometry

Graphite Furnace Atomic Absorption Spectrometry (GFAAS) was applied for quantitative analysis of selenium in various samples of cell media and FBS charges. A 5100PC system from Perkin Elmer with Zeeman background correction and the graphite furnace HGA-600 with pyrolytically coated graphite tubes with L'vov platform (PerkinElmer, Überlingen, Germany) were used.

The parameters of the measurement are shown in Table 3.

	Selenium
Source of radiation	EDL
Wave length	196.0 nm
Matrix modifier	$PdCl_2$ (10 µl, 1 g/L)
Pre atomisation temperature	1150 °C
Atomisation temperature	2200 °C
Calibration	Standard addition

Table 3 Parameters for the selenium determination by GFAAS (EDL: electrodeless discharge lamps. $PdCl_2$: palladium chloride).