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RESEARCH PAPER

Differential effects of selenium and knock-down of glutathione peroxidases on TNFα and flagellin inflammatory responses in gut epithelial cells

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Abstract Selenium (Se) is essential for human health. Despite evidence that Se intake affects inflammatory responses, the mechanisms by which Se and the selenoproteins modulate inflammatory signalling, especially in the gut, are not yet defined. The aim of this work was to assess effects of altered Se supply and knock-down of individual selenoproteins on NF-κB activation in gut epithelial cells. Caco-2 cells were stably transfected with gene constructs expressing luciferase linked either to three upstream NF-κB response elements and a TATA box or only a TATA box. TNFα and flagellin activated NF-κB-dependent luciferase activity and increased IL-8 expression. Se depletion decreased expression of glutathione peroxidase1 (*GPX1*) and selenoproteins H and W and increased TNFα-stimulated luciferase activity, endogenous IL-8 expression and reactive oxygen species (ROS) production. These effects were not mimicked by independent knock-down of either *GPX1*, selenoprotein H or W; indeed, *GPX1* knock-down lowered TNFα-induced NF-κB activation and did not affect ROS levels. *GPX4* knock-down decreased NF-κB activation by flagellin but not by TNFα. We hypothesise that Se depletion alters the pattern of expression of multiple selenoproteins that in turn increases ROS and modulates NF-κB activation in epithelial cells, but that the effect of *GPX1* knock-down is ROS-independent.

Keywords Selenoprotein · Inflammatory signalling · NF-κB · GPx1 · GPx4 · Selenium

Introduction

The micronutrient selenium (Se) is essential for human health (Bellinger et al. 2009; Fairweather-Tait et al. 2011). Low Se status has been reported to be associated with increased risk of colorectal cancer (CRC) whereas in contrast higher Se intake and status are associated with lower risk of colonic adenoma recurrence (Russo et al. 1997; Jacobs et al. 2004; Rayman 2005; Peters and Takata 2008). Supplementation with 200 µg Se per day resulted in reduced CRC mortality, especially in those individuals with low Se status prior to supplementation (Clark et al. 1996). However, the biochemical and cellular mechanisms that link Se intake gut epithelial cell function and carcinogenesis are poorly understood.

In humans, Se is incorporated as the amino acid selenocysteine into ~25 selenoproteins (Bellinger et al. 2009) that play important roles in cell protection mechanisms. The selenoproteins include a family of glutathione peroxidases (GPx1, GPx2 and GPx4) that protect cells from reactive oxygen species (ROS), the thioredoxin reductases (TR) that function in redox control (Brigelius-Flohé 2006; Arnér 2009) and members of a novel series of thioredoxin-like proteins that have been proposed to have antioxidant functions (Bellinger et al. 2009). There is evidence that Se has anti-inflammatory effects in macrophages and immune cells (Hoffmann and Berry 2008; Vunta et al. 2008) and Se supplementation has been found to modulate activation of

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the transcription factor NF-κB, which plays a pivotal role in regulation of inflammatory pathways (Jeong et al. 2002; Zamamiri-Davis et al. 2002; Prabhu et al. 2002; Gasparian et al. 2002, Christensen et al. 2007; Vunta et al. 2007).

However, critically, the in vitro effects of Se supplementation on NF-κB signalling are distinct in different cell lines. Thus, for example, supplementation of the cell culture medium lowered activation of NF-κB in human bronchial and prostate cells (Gasparian et al. 2002, Christensen et al. 2007) but had no effect on NF-κB translocation in human endothelial cells and led to increased NF-κB translocation and increased NF-κB response in macrophages (Prabhu et al. 2002; Vunta et al. 2007). These observations correspond closely to the suggestion that in vivo NF-κB pathways are regulated in a cell-type dependent mechanism (Smale 2011). Gut epithelial cells such as the Caco-2 cell line respond to bacterial and host inflammatory challenges by activation of NF-κB pathways (Kelly et al. 2004), but the NF-κB signalling response of such cells to altered Se supply or changes in selenoprotein expression are not known. Indeed, the effects of Se on inflammatory signalling in the gut and the roles of selenoproteins, especially in the epithelial cells, have not yet been defined.

However, transcriptomic analysis of samples from a recent mouse experiment examined the response of mouse colon gene expression to sub-optimal Se intake and identified the expression of genes associated with the NF-κB signalling pathway as being sensitive to dietary Se (Kipp et al. 2009). In view of the links between gut inflammation and susceptibility to colorectal cancer (Klampfer 2011), and the possible benefits of increased Se intake (Russo et al. 1997; Jacobs et al. 2004; Rayman 2005; Bellinger et al. 2009; Fairweather-Tait et al. 2011), it is important to elucidate whether Se supply affects NF-κB signalling in gut epithelial cells. The aim of the present work was to use an in vitro model of the gut epithelium to test the hypothesis that alteration of Se supply modulates NF-κB activation in response to host and microbial factors, and the to test whether the effects are due to changes in selenoprotein expression.

Materials and methods

Cell culture

Human colon adenocarcinoma Caco-2 cells were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (with 4.5 g/l glucose and Glutamax) supplemented with 10% (v/v) foetal calf serum (Sigma, Poole, UK) and 1% (v/v) (100 μg/ml) penicillin–streptomycin. For siRNA knockdown experiments, cells were grown in the same medium. In Se depletion/supplementation experiments, cells were transferred to serum-free medium containing 0.1% (v/v) penicillin–streptomycin, 1% (v/v) (100 units/ml) non-essential amino acids (Gibco), insulin (5 μg/ml) and transferrin (5 μg/ml) without (selenium-deficient medium) or supplemented with 7 ng/ml sodium selenite (equivalent to 40 nM; selenium-repleted medium), as described previously (Pagmantidis et al. 2005; Crosley et al. 2007). Cells were cultured in Se-deficient or Se-supplemented medium for 3 days and then tested for responses to treatment with either 20 ng/ml TNFα (Sigma) for or 100 ng/ml *Salmonella typhimurium* flagellin for 0–8 h.

Luciferase constructs and transfection

Luciferase constructs were obtained by modification of a previously established construct (Carlsen et al. 2002), which contains three NFκB binding sites and a TATA box linked to a luciferase coding sequence (CDS). The entire 3× NF-κB-TATA box-luciferase CDS sequence was isolated by *Hind III/ApaI* restriction digestion and ligated into pBLUE-TOPO plasmid vector (Invitrogen). The control construct was obtained by isolation of TATA box-luciferase CDS sequence by *BamHI/ApaI* restriction digestion and fusion with pBLUE-TOPO vector. The correct insertion of the 3× NF-κB-luciferase and TATA-luciferase sequences were verified by sequencing (data not shown). Stable transfections of Caco-2 cells (1×10^6 cells/transfection) with luciferase constructs (test or control) used 2–6 μg of plasmid DNA combined with 5 μl Lipofectamine™ 2000 (Invitrogen). Stably transfected cells were selected in medium containing 750 μg/ml G418 (Sigma) for 6 weeks (medium changed every 2 days) and harvested as a mixed population.

siRNA transfection

Knock-down of expression of selenoprotein genes *GPX1*, *GPX4*, selenoprotein W (*SELW*) and selenoprotein H (*SELH*) was achieved by transient transfection of Caco-2 cells with siRNA duplexes (Ambion), specific to the mRNAs of these targets The relevant siRNA sequences were: *SELH* sense GCGACCGUUGUUAUCGAGCAUUGCA and antisense UGCAAUGCUCGAUAACAACGGUCGC; *GPX1* sense GGUACUACUUAUCGAGA AUUU and antisense AUUC UCGAUAAGUAGUACCUU; SELW sense CCACCGGG UUCUUUGAAGUGAUGGU and antisense ACCAUCA CU UCAAAGAACCCGGUGG *GPX4* sense UUCGAUAUGU UCAGCAAGAUU and antisense UCUUGCUGAACAUAU CGAAUU negative control sense GUUCAAUAUUAUCAA GCGGUU and antisense CCGCUUGAUAAUAUUGAAC UU. According to Ambion's specifications, 5×10^5 cells were grown in 2 ml of serum-free medium supplemented with 5% (v/v) foetal calf serum in the absence of antibiotics.

Table 1 Primers used for RTPCR analysis of *GPX1*, *SELW*, *SELH*, *GPX4*, *GPX2*, *GAPDH* and *IL8* transcripts

Primers	Sequences	Tm
GPX4 for	5′-CGA TAC GCT GAG TGT GGT TTG C-3′	66°C
GPX4 rev	5′-CAT TTC CCA GGA TGC CCT TG-3′	29cyc
GAPDH for	5′-TGA AGG TCG GAG TCA ACG GAT TTG-3′	55°C
GAPDH rev	5′-CAT GTA AAC CAT GTA GTT GAG GTC-3′	29cyc
GPX1 for	5′-CAG TCG GTG TAT GCC TTC TCG-3′	56°C
GPX1 rev	5′-TGT CAG GCT CGA TGT CAA TG-3′	27cyc
GPX2 for	5′-GGC TTT CAT TGC CAA GTC CTT C-3′	60°C
GPX2 rev	5′-CTA TAT GGC AAC TTT AAG GAG GCG C-3′	27cyc
SELW for	5′-GTT TAT TGT GGC GCT TGA GGC-3′	60°C
SELW rev	5′-GAA CAT CAG GGA AAG ACC ACC-3′	27cyc
SELH for	5′-GCT TCC AGT AAA GGT GAA CCC G-3′	62°C
SELH rev	5′-ACC CAA ATC TCC CTA CGA CAG G-3′	27cyc
IL8 for	5′-ATG ACT TCC AAG CTG GCC GTG GCT-3′	60°C
IL8 rev	5′-TCT CAG CCC TCT TCA AAA ACT TCT C-3′	31cyc

A siRNA/transfection reagent complex was formed at 37°C by combining siRNA oligomer (30–45 nM) with 5 μl (2 μg/ml) Lipofectamine™ 2000 transfection reagent (Invitrogen) in 0.5 ml Optimem medium (Gibco), and this was applied to cells for 3 days until they were harvested. Control cells were transfected with a non-specific 'scrambled' siRNA duplex (Ambion).

Luciferase assay

NFκB activation in Caco-2 cells was determined by measuring luciferase activity in the reporter cells stably transfected with the 3× NF-κB-luciferase or TATA-luciferase constructs. Cells were washed twice in 1× PBS, mixed with 1× Reporter lysis buffer (Promega), frozen and thawed once three times, and lysed by vortexing vigorously for 15 s. Cell lysate was harvested as the supernatant fluid after centrifugation at 12,000*g* for 2 min at 4°C. Twenty microliters of cell lysate was mixed with 80 μl of 5× Luciferase assay reagent (Promega) and luciferase activity was measured on a TD-20e luminometer (Turner Designs). Protein concentration was quantified using the bicinchoninic acid protein assay (Sigma). Specific luciferase activity was calculated as luciferase activity (relative light units; RLU) per mg protein lysate.

RTPCR

The mRNA expression of the selenoproteins *GPX1*, *GPX2*, *GPX4*, *SELW* and *SELH*, the house-keeping gene *GAPDH* and the NFκB target gene *IL8* were determined by semi-quantitative RT-PCR using primer oligomers as listed in Table 1. Reverse transcription was carried out amplifying 1 μg total RNA using Transcriptor Reverse Transcriptase kit (Roche) and p(dT)15 primer for cDNA Synthesis (Roche) according to manufacturer's instructions. PCR was then carried out using 0.05 U/μl BIOTAQ polymerase (Bioline), 2 mM $MgCl_2$, 1 mM total dNTP, 1–2 pmol/μl primer, and cDNA template. PCR was carried out using the appropriate amplification cycles so that amplification was in the linear range (Table 1). Following separation of RTPCR products by gel electrophoresis and staining with ethidium bromide, gels were visualised on a GelDoc 1000 and the intensity of bands measured using UV Band software. mRNA levels were normalised against *GAPDH* and expressed relative to the control conditions.

ROS measurement

Total cellular ROS levels were determined using the Image-iT™ LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Invitrogen) according to manufacturer's specifications. A total of 6×10^4 or 3×10^4 cells were seeded in each well of a 96-well plate and grown in Se−/Se+ medium, or treated with *GPX1* siRNA/control siRNA, respectively. Cells were washed with 1× HBSS and incubated with 50 μl staining solution containing 25 μM 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) and 1 μM Hoechst 33342 (control staining of nucleic acid) for 30 min at 37°C. Cells were washed with 1× HBSS and ROS levels measured at 495/529 nm (for carboxy-H2DCFDA) and 350/461 nm (for Hoechst 33342) wavelengths on a BMG LABtech Fluostar Omega luminometer.

Western blotting

Total protein was extracted from Caco-2 cells by washing twice with ice-cold PBS, resuspension in PBS containing protease inhibitor, centrifugation at 150 rpm, 4°C for 5 min and then lysis in 25 mM HEPES buffer (pH 7.6) containing 3 mM $MgCl_2$, 40 mM KCL, 2 mM DTT, 5% glycerol and

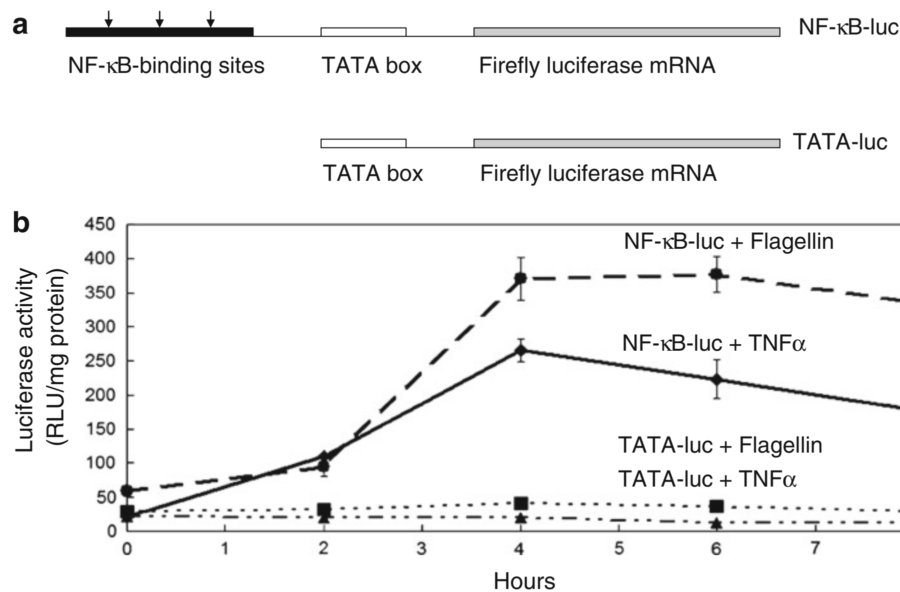

Fig. 1 Activation of NF-κB-luciferase constructs in Caco-2 cells. **a** Schematic illustration of the NF-κB-luciferase and control TATA-luciferase constructs. The NF-κB-luciferase construct contained the firefly luciferase coding region, **a** TATA box and three NF-κB binding sites whereas the NF-κB binding sites were deleted in the control. **b** Caco-2 cells were transfected with either the NF-κB-luc or TATA-luciferase construct. Stable transfectants grown in Se-supplemented medium were incubated with flagellin (100 ng/ml) or TNFα (20 ng/ml) for up to 8 h and cell extract prepared for measurement of luciferase activity. Both flagellin and TNFα elicited a stimulation of luciferase activity in the NF-κB-luciferase cells but not in the TATA-luciferase controls (TNFα *dashline with triangle* and flagellin *dashline with square*)

0.5% NP40. Protein concentration was determined using the Bradford protein assay procedure. Twenty micrograms of protein was subjected to SDS-PAGE, transferred to a PVDF membrane (ROCHE), and incubated overnight in PBS containing 5% dried milk, 0.05% Tween20 and primary antibody (Anti-GPx1 from AbCam diluted 1/500, Anti-GPx4 from Labfrontier, Korea diluted 1/500, monoclonal Anti-βactin from Sigma diluted 1/5,000). After four washes in PBS containing 0.05% Tween20, the membrane was incubated with secondary antibody (polyclonal Anti-rabbit for Anti-GPx1 and Anti-GPx4, polyclonal Anti-mouse for Anti-βactin) linked to horseradish peroxidase (from Sigma, UK diluted 1/5000) for 1 h, washed 4 times and bound antibody detected by chemiluminescence.

Statistical analysis

Groups were compared by Mann–Whitney *U*-test using SPSS 17.0 software.

Results

TNFα and flagellin activate NF-κB signalling in Caco-2 cells

Previous work has shown that it is possible to monitor activation of NF-κB signalling in vivo in transgenic mice expressing a gene in which a luciferase reporter is linked to an upstream region containing three copies of a NF-κB response element (Carlsen et al. 2002). In the present work, we adopted a similar reporter gene approach to monitor NF-κB activation in the Caco-2 gut epithelial cell line. Two gene constructs were produced (see Fig. 1a), as described in "Materials and methods". In the first, the luciferase coding region was linked to three upstream NF-κB response elements and a TATA box (NF-kB-luciferase construct). In addition, a control construct was made in which the three NF-κB response elements were absent and the region upstream of the luciferase coding sequences contained only a TATA box (TATA-luciferase construct). Caco-2 cells were transfected with either the NF-κB-luciferase construct or the control TATA-luciferase construct and stable transfectant cell populations selected. When cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, a low level of luciferase activity was detected in both transfectants (Fig. 1b, time point 0). However, as shown in Fig. 1b, addition of either TNFα or bacterial flagellin resulted in a striking $\sim$ten fold increase in luciferase activity, with the appearance of a peak activity $\sim$4 h after addition of TNFα and 4–6 h after addition of flagellin (see Fig. 1b). These results indicate that both these compounds activate NF-κB signalling in Caco-2 cells.

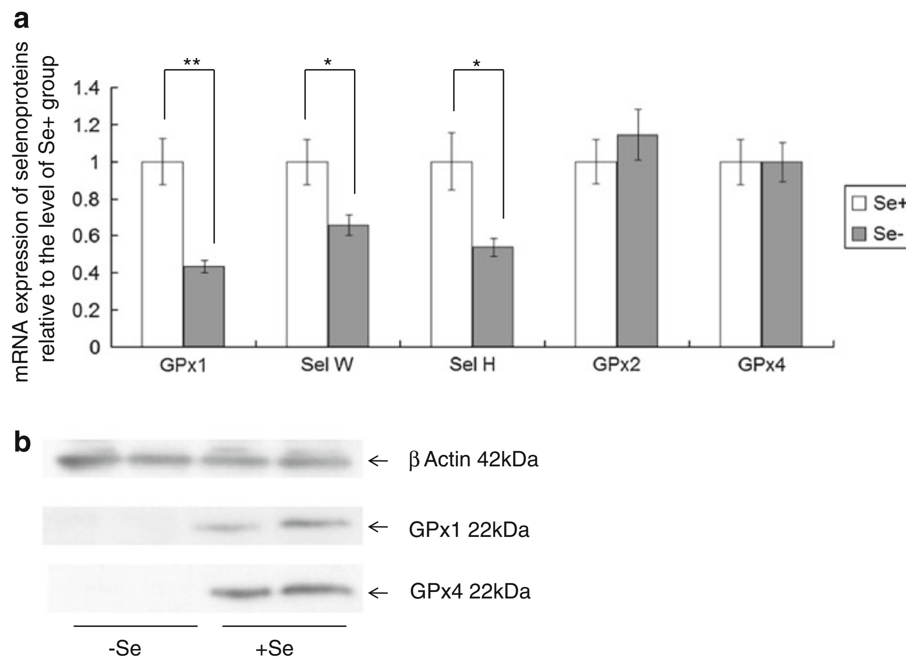

Fig. 2 Selenoprotein mRNA expression in Caco-2 cells during Se depletion. **a** Caco-2 cells were grown for 3 days in either Se-depleted or Se-supplemented (40 nM) medium, RNA extracted and mRNA levels assessed by semi-quantitative RTPCR. Values were normalised to *GAPDH* and calculated relative to the expression in the Se-supplemented group. Expression of *GPX1*, *SELH* and *SELW* was significantly lower in the Se-depleted cells, but expression of *GPX2* and *GPX4* was unaffected. $*P < 0.05$, $**P < 0.01$. **b** Western blotting of total cell protein from cells grown for 3 days in either Se-depleted or Se-supplemented medium. Note that the levels of GPx1 and GPx4 are both undetectable in Se-depleted cells

Se depletion activates NF-κB signalling in Caco-2 cells

To assess the effects of Se supply on NF-κB signalling in Caco-2 cells, cells were grown in either Se-depleted medium or medium supplemented with 40nM sodium selenite as described previously (Pagmantidis et al. 2005; Crosley et al. 2007). When grown in either medium, cells were maintained, despite the absence of serum, by the addition of insulin and transferrin and effects of Se supplementation could be specifically detected since the two media differed only in their Se content. Expression of *GPX1*, *SELW* and *SELH*, known to be very sensitive to Se supply (Pagmantidis et al. 2005; Kipp et al. 2009), was determined by semi-quantitative RTPCR. As shown in Fig. 2, expression of *GPX1*, *SELW* and *SELH* was significantly decreased in cells grown under Se-deficient conditions compared with those grown in Se-supplemented medium, indicating that the Caco-2 cells grown in Se-deficient or Se-supplemented media differ in Se status. In contrast, there was no significant change in expression of *GPX2* [known to be maintained in low Se conditions (Pagmantidis et al. 2005; Kipp et al. 2009)] in response to alterations in Se supply (Fig. 2). In addition, GPx1 and GPx4 protein expression was undetectable by Western blotting in cells grown in Se-deficient medium (Fig. 2b).

To determine whether changes in Se supply affect NF-kB signalling, luciferase activity was measured in cell extracts from cells grown under Se-supplemented or deficient conditions. As shown in Fig. 3, in cells transfected with the NF-κB-luciferase and grown in Se-deficient conditions, the increase in luciferase activity following TNFα treatment was 30% greater compared with cells grown in Se-supplemented medium ($P < 0.05$; see Fig. 3). However, there was no change in luciferase activity in response to addition of flagellin. In addition, control TATA-luciferase cells showed no alteration in the response in luciferase activity after addition of either activator whether cells were grown in Se-deficient or Se-supplemented medium.

Expression of the cytokine interleukin-8 (IL-8) has been shown to be controlled by NF-κB (Kunsch and Rosen 1993; Kelly et al. 2004). To determine whether changes in Se supply affect NF-κB-induced expression, we measured IL-8 mRNA expression in Caco-2 cells in response to TNFα under Se-deficient and Se-supplemented conditions. As shown in Fig. 4, addition of 20 ng/ml TNFα to the culture medium of NF-κB-luciferase cells led to an increase in IL-8 expression as assessed by semi-quantitative RT–PCR, and this increase was significantly larger ($P < 0.001$) under Se-deficient conditions compared with Se-supplemented conditions. This increase in IL-8

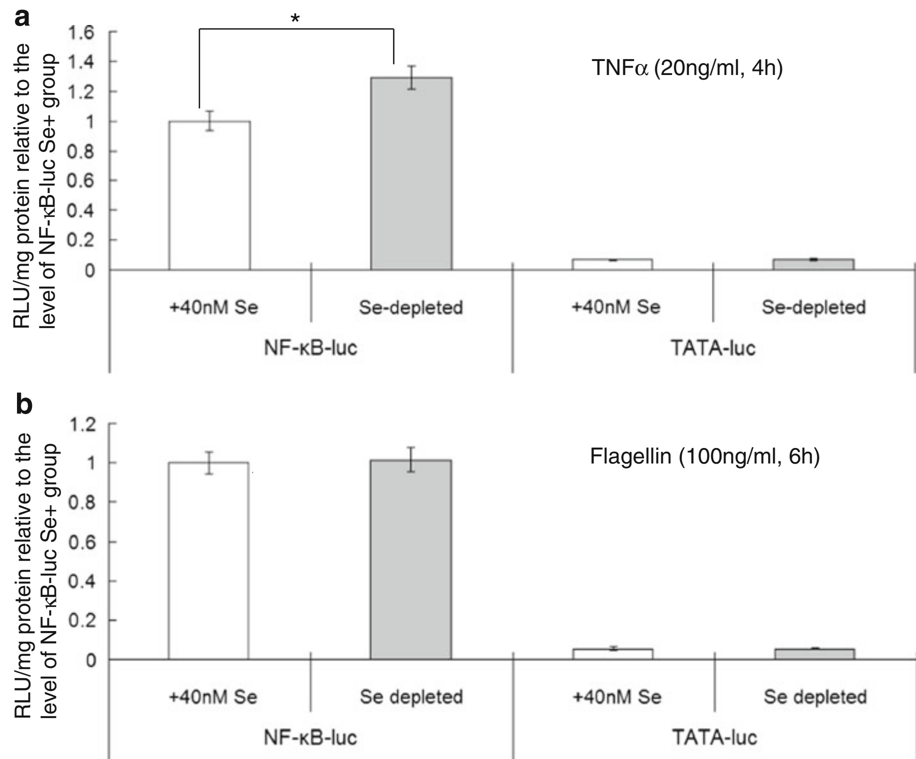

Fig. 3 Effect of Se depletion on TNFα and flagellin stimulated NF-κB-driven reporter activity. Stably transfected Caco-2 cells were grown for 3 days in either Se-depleted or Se-supplemented (40 nM) medium and then stimulated with **a** 20 ng/ml TNFα for 4 h or **b** 100 ng/ml flagellin for 6 h. Luciferase activity was measured in cell extracts, calculated per mg cell protein and expressed relative to the activity in the Se-supplemented NF-κB-luciferase cells. $^{*}P < 0.05$

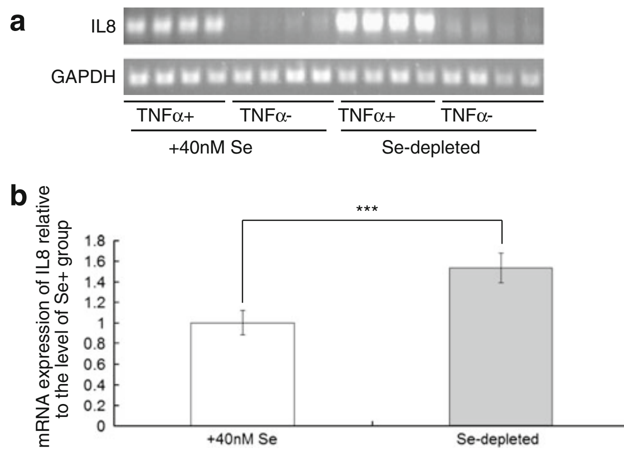

Fig. 4 Effects of Se depletion on interleukin 8 expression. **a** Semi-quantitative RTPCR amplification of NF-κB target gene *IL8* and housekeeping gene *GAPDH*. Cells were grown in Se-depleted or 40 nM Se-supplemented culture medium (3 days) and either stimulated with TNFα for 1 h or treated with carrier. **b** Quantification of *IL8* mRNA levels in cells grown in Se+ and Se− media, normalised against GAPDH and related to expression in the Se-supplemented cells. $^{***}P < 0.001$

response under Se depletion conditions is compatible with the data from the luciferase reporter experiments that indicate increased activation of NF-κB (Fig. 3a). On the contrary, in the absence of TNFα, changes in Se supply had no effect on IL8 expression.

GPX1 knock-down compromises TNFα-mediated activation of NF-κB signalling

To investigate whether these effects of Se depletion on NF-κB signalling could be accounted for by lower selenoprotein expression, *GPX1*, *SELW* and *SELH* were knocked down by addition of 80 pmol siRNA. Knock-down of *SELW* or *SELH* expression by 40–60%, respectively, (Fig. 5a, b) had no significant effect on the luciferase response of NF-κB-luciferase cells to 20 ng/ml TNFα and no effect on the luciferase activity in TATA-luciferase cells (Fig. 5c). Thus, the effects of Se depletion on the NF-κB response to TNFα do not appear to be due to the reduction of *SELW* and *SELH* expression known to occur in colonic cells and tissue in Se depletion. Similarly, transfection with an siRNA against *GPX1* did not increase the luciferase response to 20 ng/ml TNFα. Thus, the effects of Se depletion on the NF-κB-driven luciferase response to TNFα cannot be explained as being due to effects of Se depletion on expression of *GPX1*, *SELW* or *SELH*.

On the contrary, transfection with 80 pmol siRNA against *GPX1* resulted in approximately 60% decrease in GPx1 expression and a 20% decrease in the response of luciferase activity to TNFα stimulation in NF-κB-luciferase cells compared with the addition of a scrambled control siRNA (see Fig. 5c). Quantification of IL-8 expression after TNFα stimulation of Caco-2 cells showed a reduced cytokine response after *GPX1*-knock-down compared with

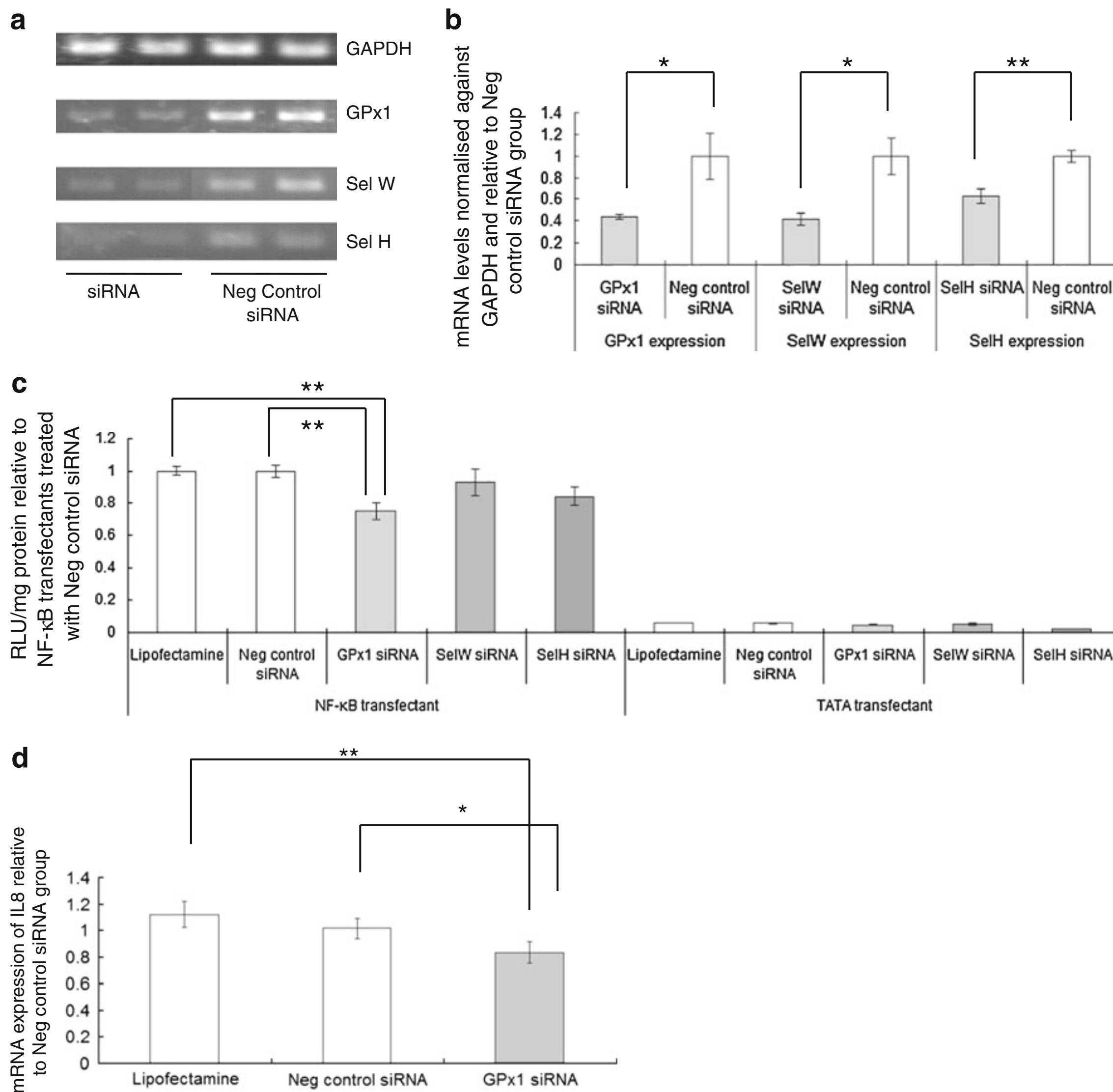

Fig. 5 Effect of selenoprotein knock-down on TNFα stimulated NF-κB-driven reporter activity and interleukin 8 expression. **a** Semi-quantitative RTPCR amplification of mRNA levels for *GPX1*, *SELW* and *SELH* after knockdown of the respective gene with 80 pmol siRNA and in control cells. Total RNA was extracted, RTPCR carried out and the products separated by gel electrophoresis. **b** The intensity of bands corresponding to the amplified products was measured using UV Band software, normalised to the housekeeping gene *GAPDH* and expression then calculated relative to the cells treated with the negative control siRNA negative control. **c** Luciferase activity was measured in NF-κB-luc transfectant or TATA-luc transfectant Caco-2 cells grown in Se-supplemented medium but after *GPX1*, *SELW* or *SELH* siRNA knockdown and stimulation with 20 ng/ml TNFα for 4 h. Luciferase activity was measured in cell extracts, calculated per mg cell protein and expressed relative to the activity in the NF-κB-luciferase cells treated with negative control siRNA. $*P < 0.05$, $**P < 0.01$. **d** Cells were either stimulated with TNFα for 1 h or treated with carrier (phosphate-buffered saline). RNA was extracted and subjected to semi-quantitative RTPCR amplification of NF-κB target gene *IL8* and housekeeping gene *GAPDH*. After separation of the amplified products by gel electrophoresis, intensity of bands was measured using UV Band software. *IL8* mRNA levels were quantified, normalised against *GAPDH* and expression related to that in the cells treated with the negative control siRNA. $*P < 0.05$, $**P < 0.01$

the response in cells treated with the control scrambled siRNA (Fig. 5d). It thus appears that GPx1 is required for the full NF-κB response to TNFα.

ROS, particularly superoxide radicals and hydrogen peroxide, are known to regulate NF-κB signalling pathways (Schreck et al. 1991; Li et al. 2001; Kabe et al. 2005). One possible explanation for the different effects of Se depletion and *GPX1* knockdown on NF-κB activation is that the two treatments have dissimilar effects on superoxide levels and the ability to maintain redox homoeostasis because of the continued activities of other selenoproteins in the knock-down experiments but not in Se-depleted cells. Therefore, the effects of both Se depletion and *GPX1* knock-down on total cellular ROS levels were investigated. As shown in Fig. 6, Se depletion led to an increase in baseline ROS levels in Caco-2 cells but *GPX1* knock-down

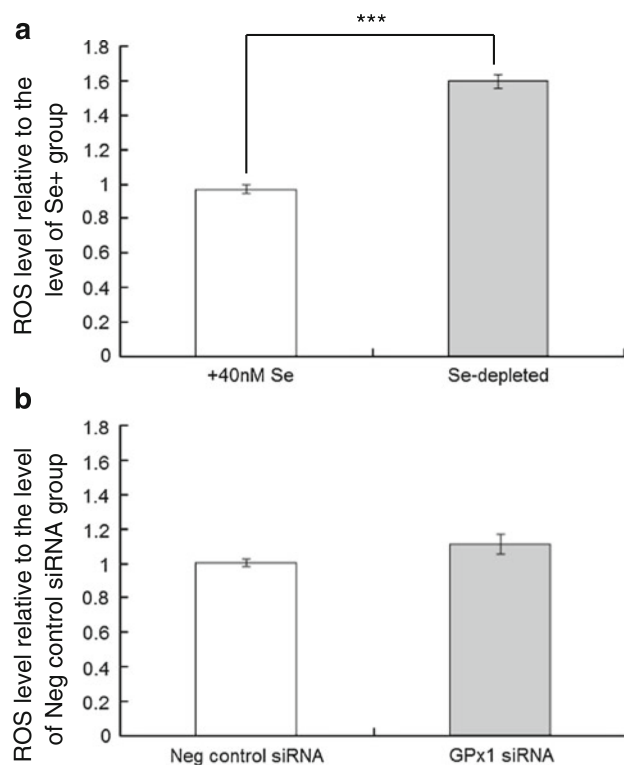

Fig. 6 Effects of Se depletion and *GPX1* knock-down on levels of reactive oxygen species in Caco-2 cells. ROS levels were determined in extracts of Caco-2 cells using carboxy-H2DCFDA fluorescent indicator. **a** Comparison between cells grown in Se-depleted and 40 nM Se-supplemented medium. **b** Comparison between cells grown in Se-supplemented medium but treated with 80 pmol negative control siRNA or *GPX1* siRNA.***$P < 0.001$

had no detectable effect. Additionally, *GPX1* knock-down was found to have no effect on expression of *GPX2* and *GPX4* mRNAs (Fig. 7); the lack of effect of *GPX1* knockdown on ROS is likely due to the ability of these selenoproteins to compensate for the lower *GPX1* expression.

GPX4 knock-down compromises flagellin-mediated activation of NF-κB signalling

Since GPx4 has been implicated in lipid hydroperoxide and leukotriene metabolism (Brigelius-Flohé 2006; Bellinger et al. 2009), we tested whether knock-down of *GPX4* affected NF-κB signalling. Gene knock-down with a *GPX4*-specific siRNA led to a 50% decrease in *GPX4* expression as assessed by RTPCR (Fig. 8a), but knock-down of this selenoprotein had no effect on the response of luciferase activity to TNFα stimulation in NF-κB-luciferase cells compared with addition of a scrambled control siRNA (results not shown). In contrast, *GPX4* knock-down caused a reduction in flagellin-activated luciferase activity and IL-8 expression (Fig. 8b, c).

Discussion

Results from both luciferase reporter assays and measurement of endogenous IL-8 expression showed that both TNFα and flagellin elicited an activation of NF-κB signalling in the gut epithelial Caco-2 cell line. This confirms earlier data showing that these enterocytic cells activate inflammatory pathways in response to such molecules (Kelly et al. 2004). In addition, the present data show that the response of NF-κB activation to TNFα was modulated by Se supply such that Se depletion led to increased luciferase activity in cells expressing the reporter driven by NF-κB response elements and to increased expression of endogenous IL-8, a chemokine that is released from epithelial cells and is key in determining neutrophil chemotaxis (Eckmann et al. 1993). These observations are consistent with recent transcriptomic data that suggest that a marginal dietary Se deficiency in the mouse causes altered expression of genes in inflammatory NF-κB signalling pathways in the colon (Kipp et al. 2009) but in addition provide the first direct evidence that NF-κB activation in a gut epithelial cell line is sensitive to Se depletion/supplementation. Moreover, Se depletion affected the response of NF-κB activation to TNFα, but not the response to bacterial flagellin, indicating modulation of responses to endogenous inflammatory mediators rather than modulation of the responses to exogenous bacterial activators via Toll-like receptors.

Earlier studies have shown that alterations in Se supply lead to changes in activation of the transcription factor NF-κB but that the effects differ between cell lines, with increased NF-κB response to Se supplementation in macrophages (Zamamiri-Davis et al. 2002; Prabhu et al. 2002; Youn et al. 2008) but lower activation of NF-κB in human bronchial and prostate cells (Jeong et al. 2002; Gasparian et al. 2002; Christensen et al. 2007). The present results show that in gut epithelial cell lines, the effect of Se on the NF-κB response to TNFα is comparable to that in bronchial and prostate epithelial cell lines and distinct from that found in macrophages, a finding consistent with the view that regulation of NF-κB differs between epithelial and immune cell types (Pasparakis 2009; Smale 2011).

Expression of *GPX1*, *SELH* and *SELW* in the mouse colon and Caco-2 cells has been shown to be highly sensitive to Se depletion (Pagmantidis et al. 2005; Kipp et al. 2009). However, knock-down of expression of these selenoprotein genes either failed to affect the response to of NF-κB activation to 20 ng/ml TNFα (*SELH*, *SELW*) or in the case of *GPX1* knock-down, it caused a reduction in the NF-κB-dependent response. Indeed, the data in Figs. 3 and 5 show opposite effects of Se depletion and *GPX1* knock-down on the NF-κB response despite both treatments lowering *GPX1* expression (see Fig. 2; Pagmantidis et al.

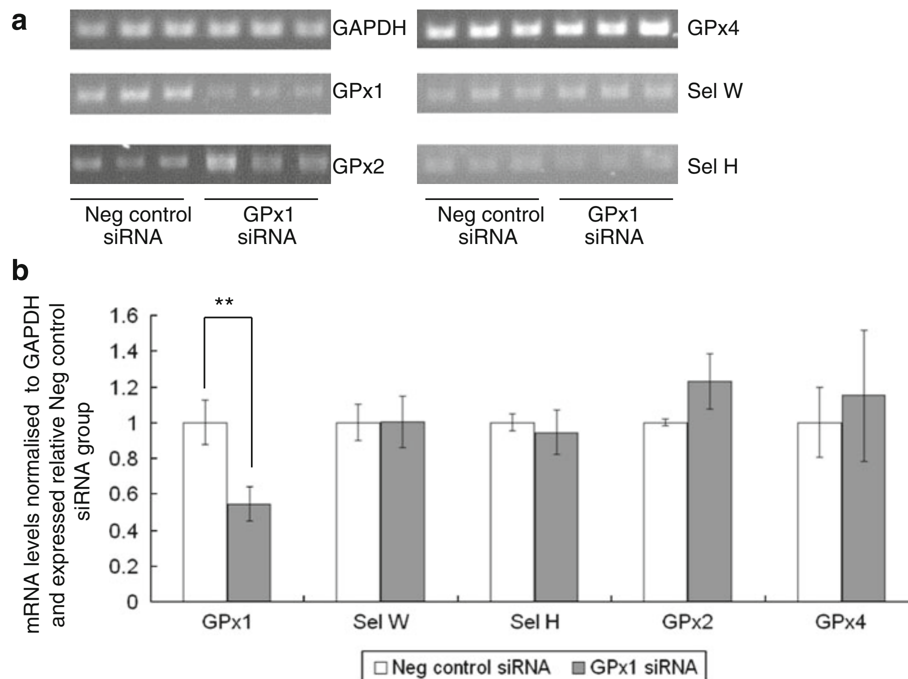

Fig. 7 Selenoprotein mRNA expression in Caco-2 cells after *GPX1* siRNA treatment. **a** Caco-2 cells grown in Se-supplemented medium were treated with 80 pmol *GPX1* siRNA or negative control siRNA for 48 h, RNA extracted and selenoprotein mRNA levels assessed by semi-quantitative RTPCR. **b** After separation of the RTPCR products by gel electrophoresis, the intensity of bands corresponding to the amplified products was measured using UV Band software. Selenoprotein mRNA expression was normalised to *GAPDH* and calculated relative to the expression in the negative control siRNA group. Expression of *GPX1* was significantly lower in *GPX1* siRNA treated cells but expression of *SELH*, *SELW*, *GPX4* and *GPX2* was unaffected. $^{**}P < 0.01$

2005). Thus, the effect of Se deficiency on NF-κB activation cannot be accounted for by a reduction of expression of *SELH*, *SELW* or *GPX1* individually.

Measurement of total Caco-2 cell ROS levels indicated that Se depletion resulted in an increase in ROS levels, but there was no increase after *GPX1* knock-down. A key difference between *GPX1* knock-down and Se depletion is that Se depletion alters the expression of multiple selenoproteins (Fig. 2), but that knock-down of *GPX1* does not (Fig. 7). Thus, after *GPX1* knock-down when Se supply is adequate, expression of these other selenoproteins is expected to be maintained and we speculate that their activity can compensate for the lower *GPX1* expression; as a result, any elevation of a particular species of oxidative signal, such as superoxide radicals, will be transient and rapidly corrected, whereas in Se-depleted cells, the rise in ROS cannot be corrected. Superoxide radicals are known to modulate inflammatory signalling pathways, and activation of NF-κB is redox sensitive (Schreck et al. 1991; Takada et al. 2003; Brigelius-Flohé et al. 2004; Kabe et al. 2005) with modulation of hydrogen peroxide clearance affecting IKK activity (Li et al. 2001). Therefore, our hypothesis is that in gut epithelial cells, the TNFα-induced, IKK-mediated activation of NF-κB following Se depletion is modulated by increased superoxide levels as a result of Se supply altering expression of multiple selenoproteins.

In contrast, when Se supply was adequate, knock-down of *GPX1* expression led to lower NF-κB activation in response to TNFα but had no effect on baseline ROS levels. This suggests that GPx1 can have a ROS-independent effect on TNFα-induced activation of NF-κB. ROS-independent activation of NF-κB has been reported in other cell types (Legrand-Poels et al. 1997), and knock-out of *GPX1* has opposite effects on ROS-dependent and reactive nitrogen species-dependent signalling pathways (Fu et al. 2001). In addition, *GPX4* knock-down affected activation of NF-κB by bacterial flagellin, suggesting that changes in GPx4 expression modulate interactions between bacterial microbiota and the host. Thus, the results indicate that in Caco-2 cells, both GPx1 and GPx4 are both required for full response of NF-κB, but it appears that GPx1 modulates pathways operating through the TNFα receptor and GPx4 pathways through Toll-like receptor 5 in response to flagellin. These differences in response of NF-κB activation after *GPX1* and *GPX4* knock-down may reflect their different products derived from lipid peroxides (Seiler et al. 2008).

In conclusion, the present results indicate that Se depletion affects the response of NF-κB activation to

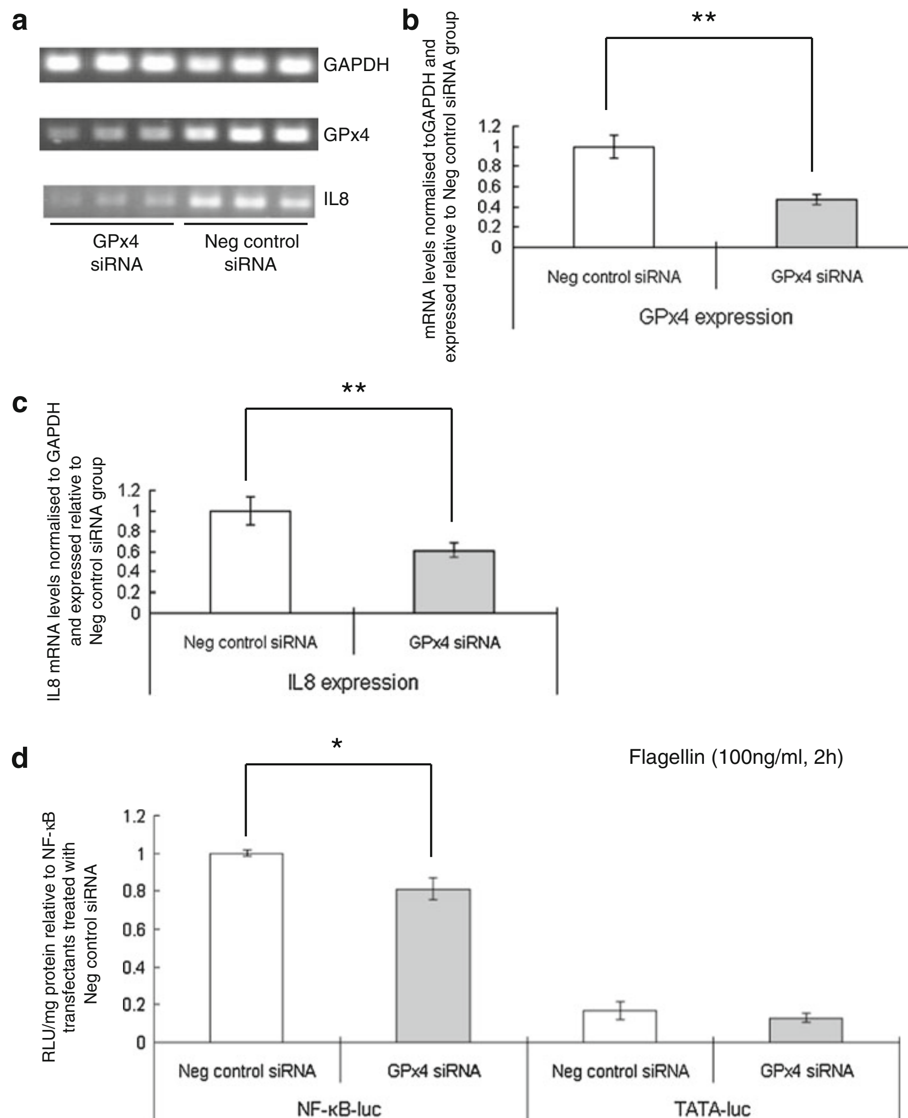

Fig. 8 Effect of *GPX4* knock-down on flagellin stimulated NF-κB-reporter activity and endogenous interleukin 8 expression. **a** Semi-quantitative RTPCR amplification of *GPX4* mRNA in cells treated with either a specific *GPX4* siRNA (120 pmol) or the negative control. Total RNA was extracted, RTPCR carried out and the products separated by gel electrophoresis. **b** The intensity of bands corresponding to the amplified *GPX4* product was measured using UV Band software, normalised to the housekeeping gene *GAPDH* and expression then calculated relative to the cells treated with the negative control siRNA. **c** Quantification of IL8 expression by semi-quantitative RTPCR. Bands corresponding to the amplified *IL8* products were normalised to the housekeeping gene *GAPDH* and expression then calculated relative to the cells treated with the negative control siRNA. **d** Luciferase activity measured in NF-κB-luc transfectant or TATA-luc transfectant Caco-2 cells after *GPX4* siRNA knockdown and stimulation with 100 ng/ml flagellin for 2 h. Luciferase activity was measured in cell extracts, calculated per mg cell protein and expressed relative to the activity in the NF-κB-luciferase cells treated with negative control siRNA. *$P < 0.05$, **$P < 0.01$

TNFα, but not the response to bacterial flagellin, and that *GPX1* knock-down and *GPX4* knock-down have distinct effects on activation of NF-κB. We hypothesise that Se depletion affects the pattern of expression of multiple selenoproteins and that the combined changes in expression lead to increased superoxide levels that in turn modulate NF-κB signalling. In contrast, the effect of *GPX1* knock-down alone is ROS-independent. The complex roles of selenoproteins in modulating these inflammatory pathways is further illustrated by the finding that knock-down of *GPX4* lowers flagellin-induced activation of NF-κB but has no effect on TNFα-induced activation. Effects of Se on inflammatory signalling in macrophages have been linked both to effects of 15-deoxy-delta12,14 prostaglandin J_2 and to Toll-like receptor pathways and IκB-kinaseß (Vunta et al. 2007; Youn et al. 2008), and so future work on gut epithelial cell lines should explore the possible links between Se, selenoproteins, pathways of activation of

NF-κB and eicosanoid metabolism but bearing in mind that regulation of NF-κB differs between epithelial and immune cell types (Pasparakis 2009). Since gut inflammation has been found to be associated with subsequent tumour development (Klampfer 2011), these effects of Se supply and selenoproteins on inflammatory pathways in gut epithelial cells provide a potential mechanistic link between the effects of Se intake on susceptibility to colorectal cancer, inflammation and carcinogenesis.

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