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GPx2 Counteracts PGE_2 Production by Dampening COX-2 and mPGES-1 Expression in Human Colon Cancer Cells

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Abstract

GPx2, the gastrointestinal glutathione peroxidase, is a selenoprotein predominantly expressed in the intestine. An anti-inflammatory and anticarcinogenic potential has been inferred from the development of colitis and intestinal cancer in GPx1 and GPx2 double knockout mice. Further, induction by Nrf2 activators classifies GPx2 as a protective enzyme. In contrast, enhanced COX-2 expression is consistently associated with inflammation. The antagonistic roles and an intriguing co-localization of GPx2 and COX-2 prompted us to investigate their possible mutual regulation. Both enzymes were upregulated in tissues of patients with colorectal cancer and colitis, and co-localized in the endoplasmic reticulum. A stable knockdown of GPx2 in HT-29 cells by siRNA resulted in a high basal and IL-1-induced expression of COX-2 and mPGES-1, enzymes required for the production of the pro-inflammatory PGE_2. Accordingly, si-GPx2 cells released high concentrations of PGE_2. Observed effects were specific for GPx2, since COX-2 and mPGES-1 expression was not affected by selenium-deprivation which resulted in the disappearance of GPx1. It is concluded that GPx2 by compartmentalized removal of hydroperoxides silences COX-2 activity and suppresses PGE_2-dependent COX-2 expression. Thus, GPx2 may prevent undue responses to inflammatory stimuli and, in consequence, inflammation-driven initiation of carcinogenesis. *Antioxid. Redox Signal*. 10, 1491–1500.

Introduction

Out of the about 30 selenoproteins present in humans (28), five belong to the family of glutathione peroxidases (GPxs) (4, 6). Although all GPxs reduce hydroperoxides, their physiological functions appear to be different. Whereas GPx1 is likely the most efficient antioxidant device but dispensable for survival, GPx4 (PHGPx) is mandatory for male fertility (42) and essential for embryonic development (21). The gastrointestinal glutathione peroxidase (GPx2) was originally described to be exclusively expressed in the gastrointestinal system and was, therefore, believed to build a barrier against food-borne or endogenously produced hydroperoxides (8). However, its particular distribution pattern suggests a role in the regeneration of the intestinal epithelium. Its level is highest in the crypt grounds where proliferation takes place and declines gradually to the luminal surface where apoptosis prevails (17). Further, GPx2 is enhanced in human colorectal adenomas (17), in Barrett's esophageal mucosa (31), during the neoplastic transformation of squamous epithelial cells (38), and in lung adenocarcinomas from smokers (45). In tumorigenesis, GPx2 most likely plays a preventive role, since mice in which both GPx1 and GPx2 have been knocked out develop intestinal cancer (9), and a GPx2 knockout predisposes mice to skin cancer upon UV-irradiation (43).

Tumor prevention by GPx2 is likely due to its anti-inflammatory action (10), since intestinal cancer in GPx1/2 double knockout (DKO) mice was a rather late event that was preceded by chronic ileocolitis (13). GPx2 was also reported to be upregulated in other inflammatory conditions, for example, during the regeneration of the crypt epithelium after γ-irradiation (12), after contact with gastrointestinal bacteria (14), and in lungs of rats exposed to cigarette smoke (39). A protective role of GPx2 also complies with its transcriptional regulation by Nrf2 (1, 39), a transcription factor that usually induces cytoprotective enzymes (26).

A phenomenon consistently associated with inflammatory

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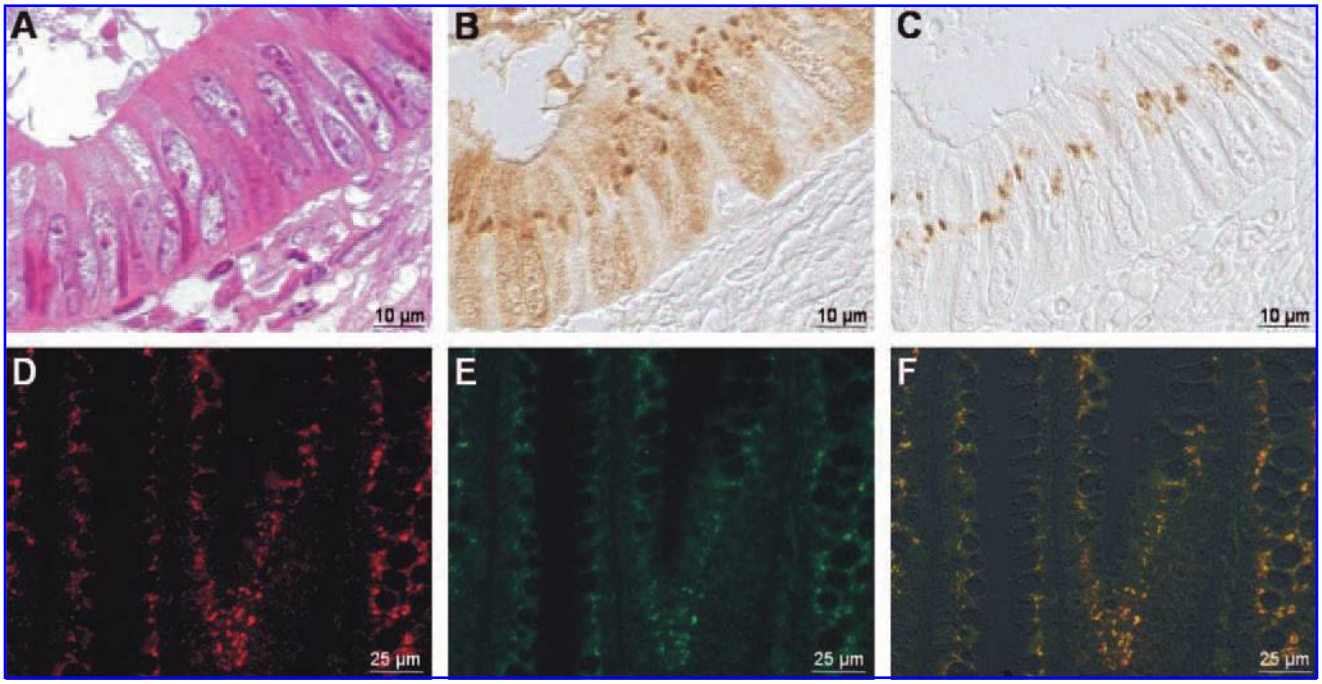

FIG. 1. Co-expression of GPx2 and COX-2 in sporadic colorectal cancer and ulcerative colitis in humans. (**A**–**C**) Cellular localization of GPx2 and COX-2 in colorectal carcinoma. Serial sections from a tumor (stage G3) stained with (**A**) H&E, (**B**) GPx2, and (**C**) COX-2 antibody. (**D**–**F**) Serial sections from a tumor (stage G2) stained with antibodies against (**D**) GPx2 and (**E**) the endoplasmic reticulum marker calnexin. (**F**) In the overlay of (**D**) and (**E**) (combined with light microscopy), the color turns *yellow*, indicating the co-localization of GPx2 and COX2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

processes is the activation of the arachidonate cascade via the inducible cyclooxygenase (COX-2) (11). COX-2 is induced by pro-inflammatory cytokines such as TNFα, IL-1α, and IL-β (37), but also in an autocrine positive feedback loop by its product PGE_2 (19). In fact, PGE_2 is considered to be a key mediator of acute inflammatory responses (36) and inflammatory bowel disease (30), as reviewed in Ref. (44). The abundant production of PGE_2 also depends on the induction of the microsomal prostaglandin E synthase (mPGES-1) which directs the common intermediate of the PG synthesis towards E-type prostaglandins. Overexpression of mPGES-1 has indeed been reported in inflammatory bowel disease (41).

Several links exist between cyclooxygenases and glutathione peroxidases: (a) lipoxygenases (LOX) and COXs require a minimum level of hydroperoxides for activity (29), and accordingly, GPx1 and GPx4 were reported to inhibit COX and LOX activities by lowering the cellular peroxide tone (4); (b) GPx4 overexpression in L929 cells inhibited basal and TNF-induced COX-2 mRNA expression as well as PGE_2 production, while PGE_2 restored the response to TNF (19); (c) both COX-2 and GPx2 are increased in inflammatory bowel disease and during cancer development. We here show that GPx2 and COX-2 co-localize in the intestinal epithelium of patients with sporadic colorectal tumors and colitis, making an interplay of both enzymes likely; stable downregulation of GPx2 by siRNA in HT-29 cells strongly enhances COX-2 and mPGES-1 expression as well as PGE_2 production; the prevention of COX-2 expression was GPx2 specific, since depletion of GPx1 and other selenoproteins by selenium deprivation did not affect COX-2 expression.

Materials And Methods

Histology and immunohistochemistry

Large intestine samples from 11 patients with sporadic colorectal cancer and from 30 patients with ulcerative colitis were collected during curative surgical treatment or colonoscopy with informed consent. Tissues were prepared for histology as described (17). Differentiation and inflammation were evaluated according to histomorphological parameters (33). In 44 mucosa samples of cancer, patients' stages of cancer were classified as morphologically normal (N, $n = 21$), and well (G1, $n = 10$), moderately (G2, $n = 3$), or poorly (G3, $n = 17$) differentiated, as well as undifferentiated (G4, $n = 6$). Usually multiple stages of differentiation were present in one sample. The inflammatory status of 70 samples of colitis patients was scored and classified as low (I1, $n = 14$), moderate (I2, $n = 41$) and high (I3, $n = 15$).

Immunohistochemistry including respective controls was performed and evaluated as described (2). Primary antibodies, rabbit anti-GPx2 (17) and rabbit anti-COX-2 (Oxford Biomedical Research, Oxford, MI), were applied overnight at 4°C. A biotin-spacer-conjugated goat anti-rabbit IgG (Jackson, West Grove, PA), followed by a streptavidin-biotin-horseradish peroxidase complex (StreptABComplex/HRP, DakoCytomation, Hamburg, Germany) and diaminobenzidine (DakoCytomation) were used for visualization. For

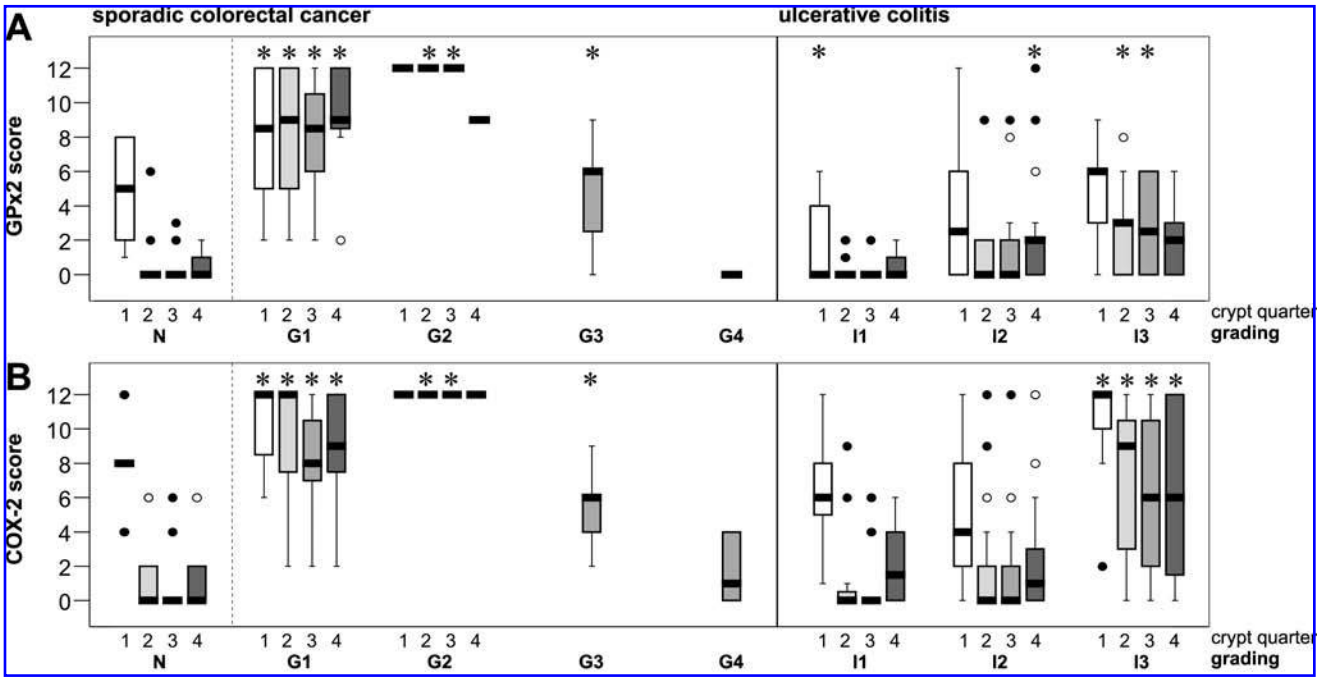

FIG. 2. Semiquantitative evaluation of immunoreactivity of membrane-bound GPx2 and COX-2. (**A**) GPx2 and (**B**) COX-2 immunoreactivity was semiquantitatively estimated (in score points) in four crypt quarters from the lumen (No. 1) to the base (No. 4) in different stages of dedifferentiation. In sporadic colorectal carcinoma (*left side*), different stages of differentiation are indicated as N = normal, G1 = well differentiated, G2 = moderately differentiated, G3 = poorly differentiated, and G4 = undifferentiated. In ulcerative colitis (*right side*), severity of inflammation is indicated as I1 = low, I2 = moderate, and I3 = high. Data are indicted as *box plots* (SPSS 11) to give an overview on the variable distribution of individual values. The *black line* in the box plot indicates the median for each group. The *box intercept* marks the distribution between the 25th and 75th percentile (comprising 50% of all values). The whiskers represent the maximum and the minimum values without containing outliers. Outliers (○) are defined as values 1.5 box lengths far from the 25^{th} and 75^{th} percentile. Extreme values (●) represent values lying outside the maximum and minimum values. $^*p < 0.05$ *vs.* morphologically inconspicuous tissue of patients with colorectal cancer.

double-labeling, GPx2 was detected with a Cy3-labeled secondary sheep anti-rabbit antibody (Sigma, Taufkirchen, Germany). The calnexin antibody (Stressgen, Victoria, Canada) was detected with a biotinylated secondary donkey anti-rabbit antibody (Jackson) and with Cy2-labeled streptavidin (Kirkegaard and Perry, Gaithersburg, MD).

GPx2 and COX-2 immunoreactivity was semiquantitatively evaluated in score points (score point = abundance x intensity of the staining). The abundance was expressed as percentage of GPx2 or COX-2 positive epithelial cells, respectively (0 = no positive cells; 1 = 1–10%; 2 = 11–50%; 3 = 51–75%; 4 = 76–100%). The intensity was expressed by four grades (0 = negative; 1 = weak; 2 = moderate; 3 = intense). Crypts were divided into quarters from the lumen (No. 1) to the ground (No. 4). Due to the loss of the typical crypt architecture in G3 and G4, crypt convolutes were assessed as a total.

Cell culture

HT-29 cells (human colon adenocarcinoma cells; DSMZ ACC 299) were grown in DMEM (high glucose) with 1% nonessential amino acids, 10% FCS (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Karlsruhe, Germany). Media of stably transfected cells contained 800 μg/ml Geneticin (Calbiochem, Bad Soden, Germany). Selenium-supplemented cell culture media contained 50 n*M* sodium selenite. Cells were generally grown ± Se for 3 days to adjust the selenium status.

siRNA-mediated knockdown of GPx2 and COX-2

Two different oligonucleotides were selected for the siRNA-mediated knockdown of GPx2 and one for the knockdown of COX-2 using the software "siRNA Wizard™ v2.4" (InvivoGen, Toulouse, France). si-GPx2#1 fwd: 5′-ACCTCGATCCTGAACAGTCTCAAGTATCAAGAG TACTTGAGACTGTTCAGGATCTT-3′; si-GPx2#1 rev: 5′-CAAAAAGATCCTGAACAGTC TCAAGTACTCTTGATACTTGAGACTGTTCAGGATCG-3′; si-GPx2#2 fwd: 5′-ACCTCGCTCAACACACAGATCTCCTATCAAGAGTAGGAGATCTGTGTGTTGAGCTT-3′; si-GPx2#2 rev: 5′-CAAAAAGCTCAACACACAGATCTCCTACTCTTGATAGGAGA TCTGTGGTTGAGCG-3′; si-COX-2 fwd: 5′-ACCTCGTGGCTATCACTTCAAACTGATCAAGAGTCAGTTTGAAGTGATAGCCACTT-3′; si-COX-2 rev: 5′- CAAAAAGTGGCTATCACTTCAAACTGACTCTTGATCAGTTTGAAGTGATAGCCACG-3′. Annealed oligonucleotides were cloned into the *Bbs I* site of psiRNA-h7SKneo. Correctness was verified by sequencing. The plasmid psiRNA-h7SKnScr (InvivoGen), containing a scramble sequence served as control. 2 x 10^5 cells were transfected with 1 μg of the corresponding plasmids by us-

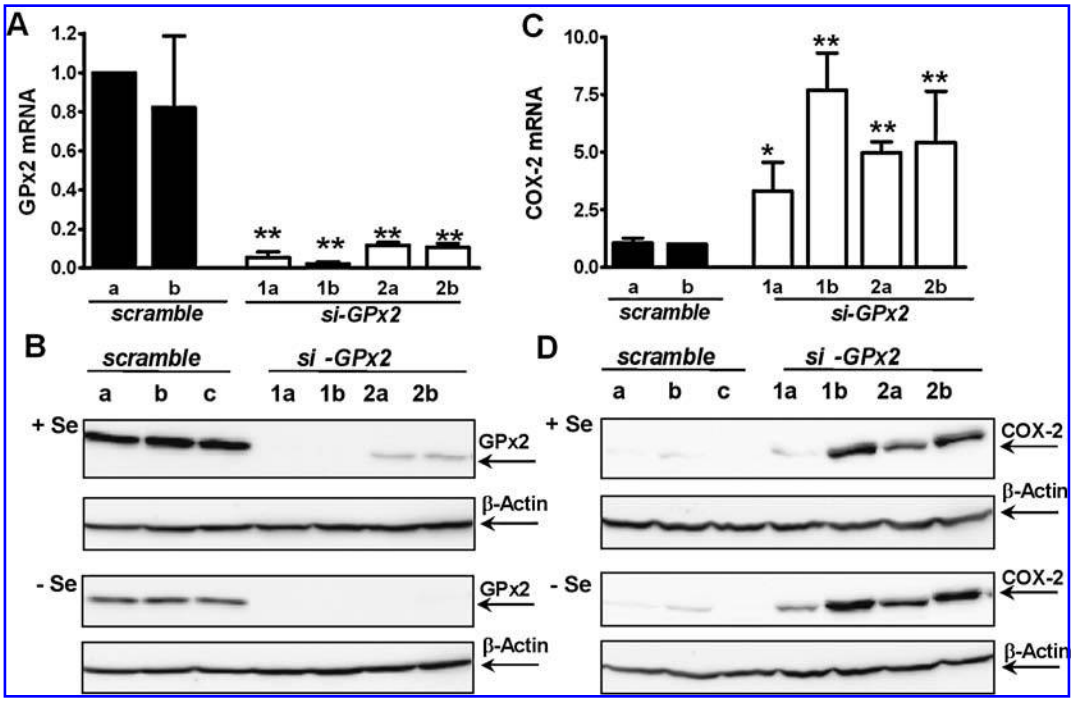

FIG. 3. GPx2 counteracts COX-2 expression in HT-29 cells. (**A**) Verification of GPx2 knockdown at mRNA level. Scramble or si-GPx2 clones were grown without selenium supplementation. GPx2 cDNA was estimated by qRT-PCR and normalized to β-actin. Scramble a was set to 1. Values are means ± SD ($n = 3$). $^{**}p < 0.01$ *vs.* scrambles. (**B**) Verification of GPx2 knockdown at protein level. Cells were grown with (+Se) or without (-Se) 50 n*M* sodium selenite for 3 days. GPx2 protein was estimated by Western blot analysis with β-actin as reference. (**C**) High levels of COX-2 mRNA in si-GPx2 clones. Cells were grown as in (**A**) and COX-2 cDNA analyzed by qRT-PCR and normalized to β-actin. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* scrambles. (**D**) High levels of COX-2 protein in si-GPx2 clones. Cells were grown as in (**B**). COX-2 protein was analyzed by Western blot analysis with β-actin as reference. Respective blots with and without selenium were run simultaneously and under identical conditions. They can, thus, be directly compared.

ing SuperFect reagent (Qiagen, Hilden, Germany). Stable transfectants were selected with 800 μg/ml Geneticin.

Western blot analysis

Cells were lysed for 15 min on ice in RIPA buffer (50 m*M* Tris, 150 m*M* NaCl, 2 m*M* EGTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40). Aliquots (50 μg protein/lane) were subjected to SDS-PAGE and blots performed as described (1). GPx2 was detected with the anti-GPx2 antibody also used for immunohistochemistry, GPx1 with a sheep anti-GPx1 antibody (Abcam, Cambridge, UK), and COX-2 with a rabbit anti-COX-2 antibody (Cayman Chemical, Ann Arbor, MI). β-Actin served as internal control (Abcam). Peroxidase-conjugated anti-rabbit or anti-sheep IgG, respectively, (Chemicon, Hofheim, Germany) were used as secondary antibodies. Detection was achieved in a Fuji LAS3000-CCD system with Supersignal West Dura (Perbio, Bonn, Germany) as substrate.

Quantitative real-time-PCR

Total RNA was isolated with the Invisorb Spin Cell RNA Mini kit (Invitek, Berlin, Germany). Reverse transcription and qRT-PCR was performed as described (32). PCR conditions were: 10 min 95°C, followed by 40 cycles of 30 sec 95°C; 1 min 60°C (GPx2, β-actin) or 62°C (COX-2, GPx1, mPGES-1); and 2 min 72°C. PCR products were quantified with a standard curve ranging from 1×10^4 to 1×10^9 copies of each amplicon. β-Actin was used as housekeeping gene. All reactions were performed in triplicate. Primer sequences were: GPx2 fwd: 5′-GTGCTGATTGAGAATGTGGC-3′; GPx2rev: 5′-AGGATGCTCGTTCTGCCA-3′; COX-2 fwd: 5′-TTCAAGACAGATCATAAGCGAG-3′; COX-2 rev: 5′-GTGGCATACATCATCAGACCA-3′; GPx1 fwd: 5′-GAAGAGATTCTGAATTCCCT CAA-3′; GPx1 rev: 5′-GAACTTCTCAAAGTT CCAGGCA-3′; mPGES fwd: 5′-ACGCTGCTGG TCATCAAGATG-3′; mPGES rev: 5′-TGGCAAA GGCCTTCTTCCGC-3′; β-actin fwd: 5′-CAAGA GATGGCCACGGCTGCT-3′ and β-actin rev: 5′-TCCTTCTGCATCCTGTCGGCA-3′.

Glutathione peroxidase activity

Cells were grown in 75 cm^2 flasks, resuspended in homogenization buffer (100 m*M* Tris, 300 m*M* KCl, 0.1% Triton X-100, pH 7.6), lysed by sonication, and centrifuged (10 min, 20,000 *g*, 4°C). GPx-activity was measured in the glutathione reductase-coupled test (5).

PGE_2 immunoassay

Cells were grown in 6-well plates and then stimulated for 24 h with IL-1β (1 ng/ml) in serum-free medium. PGE_2 was measured with a PGE_2 competitive enzyme immunoassay (Cayman Chemical) with 50 μl undiluted sample according to the manufacturer's protocol.

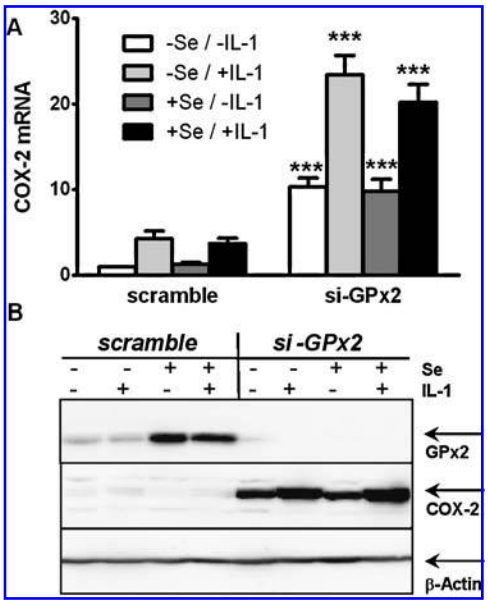

FIG. 4. Induction of COX-2 by IL-1 remains in si-GPx2 clones. (**A**) mRNA: Scramble b and si-GPx2 clone 1b were grown as indicated and stimulated or not with IL-1β (1 ng/ml) in serum-free medium for 4 h. COX-2 cDNA was quantified by qRT-PCR. COX-2 of the unstimulated selenium-deficient scramble clone was set to 1. Values are means ± SD ($n = 4$). ***$p < 0.001$ *vs.* scramble. (**B**) Protein: Cells were grown as in (**A**) and stimulated with IL-1β (1 ng/ml) for 24 h. GPx2 and COX-2 expression were analyzed by Western blot analysis.

Cellular hydroperoxides

Cells were grown in 6-well plates. Medium was changed into medium free of serum and phenol-red containing 20 μM DHR123 (Molecular Probes, Leiden, The Netherlands). After 30 min, the medium was discarded and cells were treated with 20 μM 13-hydroperoxyoctadecadienoic acid (HPODE) (15 min) in medium lacking phenol-red and serum. Cells were harvested by trypsination, counted, and suspended in 3 ml medium. Fluorescence (excitation: 485 nm, emission: 529 nm) was measured with an AB2 reader (Thermo Spectronic, Cambridge, UK). 13-HPODE was prepared as described (18).

Statistics

All experiments were conducted at least three times. Group differences were tested by one-way or two-way ANOVA, followed by Bonferroni's or Dunnett's posttests as appropriate. Statistical significance of histological values was evaluated with the Mann–Whitney U-test by SPSS software, version 11.0.

Results

GPx2 and COX-2 are co-expressed in colorectal cancer and ulcerative colitis.

GPx2 and COX-2 were distributed in a similar cellular and subcellular pattern in human colorectal cancer and ulcerative colitis. In colorectal epithelial cells, GPx2 was present in three different localizations, the nucleus, the cytoplasm, and in distinct structures capping the nucleus towards the apical side (Fig. 1B), where also COX-2 was localized (Fig. 1C). Double labeling with the endoplasmic reticulum marker calnexin was performed in colorectal tumor tissue in an early stage of malignancy where structural GPx2 predominates (17). The overlay identified the GPx2-positive structures as endoplasmic reticulum (Figs. 1D–F), the compartment of COX-2 expression (40).

The co-expression of GPx2 and COX-2 was further confirmed in serial tissue sections. Semiquantitative scoring of membrane-bound GPx2 and COX-2 revealed a high increase of both enzymes in early stages G1 and G2 of dedifferentiation (*see* 'Methods' for definition) throughout the crypt, whereas their expression gradually decreased in advanced stages G3 and G4 of malignant transformation (Fig. 2). In ulcerative colitis, GPx2 and COX-2 were upregulated in the same manner depending on the degree of inflammation with the highest expression in stage I3. Nuclear and cytosolic GPx2 immunoreactivity was increased similarly during progression of both diseases (not shown).

GPx2 counteracts COX-2 expression

Compared to other human colon cancer cell lines such as CaCo2 and SW480, HT-29 cells express high amounts of

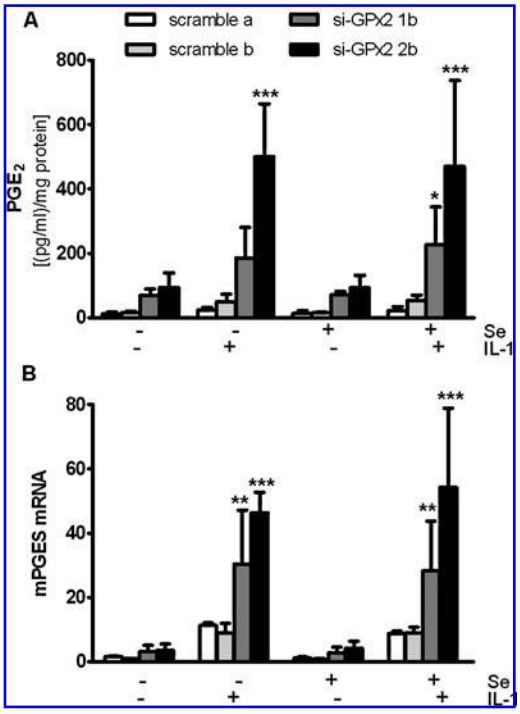

FIG. 5. Enhanced PGE_2 production and mPGES-1 expression in si-GPx2 clones. (**A**) PGE_2: cells were grown as indicated. After 3 days, medium was changed into serum-free medium containing IL-1β (1 ng/ml). Released PGE_2 was determined by ELISA after 24 h and expressed as (pg/ml)/mg protein. Values are means ± SD ($n = 4$). *$p < 0.05$, ***$p < 0.001$ vs. scrambles. (**B**) mPGES-1: cells grown as in (**A**) were stimulated with IL-1β (1 ng/ml) in serum-free medium for 4 h. mPGES-1 cDNA was amplified by qRT-PCR. Values are means ± SD ($n = 4$ for scramble b, si-GPx2 1b, si-GPx2 2b; $n = 2$ for scramble a). **$p < 0.01$, ***$p < 0.001$ *vs.* scramble b.

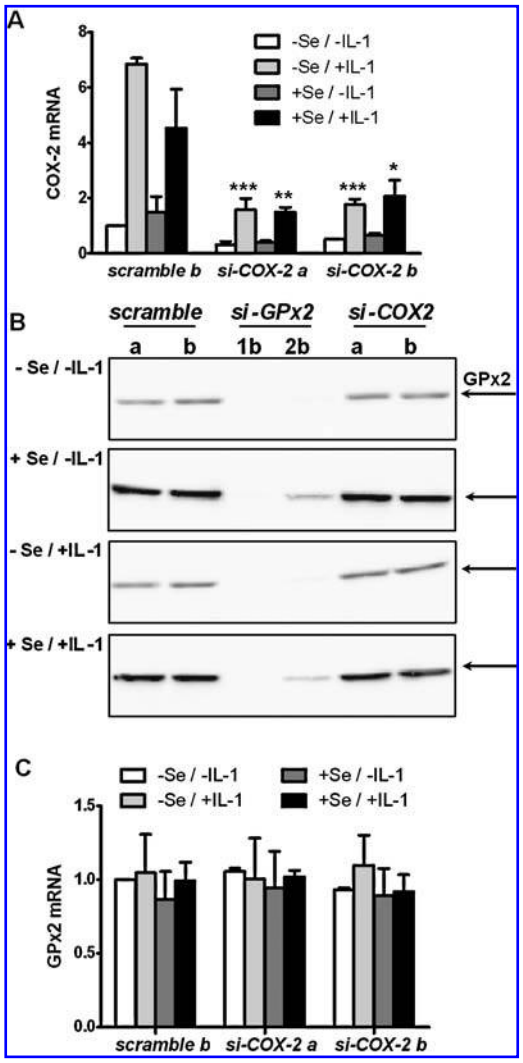

FIG. 6. COX-2 has no effect on GPx2 expression. (**A**) COX-2 mRNA expression in stable HT-29 COX-2 knockdown cells. Cells were grown as indicated. After 3 days, cells were stimulated with IL-1β (1 ng/ml) for 4 h in serum-free medium. RNA was extracted, reverse transcribed, and COX-2 was amplified by qRT-PCR. β-Actin was taken as reference. $^{*}p < 0.05$ and $^{***}p < 0.001$ *vs.* scramble-b. (**B**) GPx2 protein expression in stable HT-29 COX-2 knockdown cells. Cells were grown with (+Se) or without (-Se) 50 n*M* sodium selenite for 3 days. Thereafter, medium was changed into serum-free medium, to which IL-1 (1 ng/ml) was added for 48 h. Cells were lysed and analyzed for GPx2 by Western blot analysis. (**C**) GPx2 mRNA expression. Cells were treated as in (**A**), and GPx2 was amplified by real-time PCR. β-Actin was taken as reference. Results are representative for three (B) or two (A, C) independent experiments.

GPx2 and were, therefore, chosen as a model for testing a putative GPx2/COX-2 interaction. GPx2 was stably knocked down with two different siRNA constructs to exclude nonspecific effects. With the different siRNAs, two sets of stable transfectants were obtained, si-GPx2-1 and si-GPx2-2. A plasmid producing a scramble siRNA without any homology to human sequences was used as negative control. Three scramble (scr a–c) and four si-GPx2 (1a, 1b, 2a, 2b) clones were selected and analyzed for GPx2 expression. In all four GPx2-knockdown clones GPx2 expression was clearly suppressed, both at mRNA (Fig. 3A) and protein level (Fig. 3B). Deficiency of the selenoprotein GPx2 was observed irrespective of selenium supplementation. In clones 1a and 1b, GPx2 mRNA levels were reduced to <10% of those in scramble clones. GPx2 protein was neither detectable in the -Se nor in the +Se state. In clones 2a and 2b, GPx2 protein was absent in selenium-deficiency too, whereas tiny amounts appeared upon selenium supplementation. The amounts, however, were substantially less than in selenium-deficient scramble clones. Scramble siRNA did not affect GPx2 expression at all.

COX-2 mRNA (Fig. 3C) and protein (Fig. 3D) was nearly absent in scramble cells. In contrast, when GPx2 was knocked down, COX-2 became markedly expressed at mRNA and protein level (Fig. 3C/D). Selenium deficiency did not have a pronounced effect on COX-2 expression. This implies that even the reduced levels of GPx2 in selenium-deficient scramble clones were sufficient to suppress COX-2 ex-

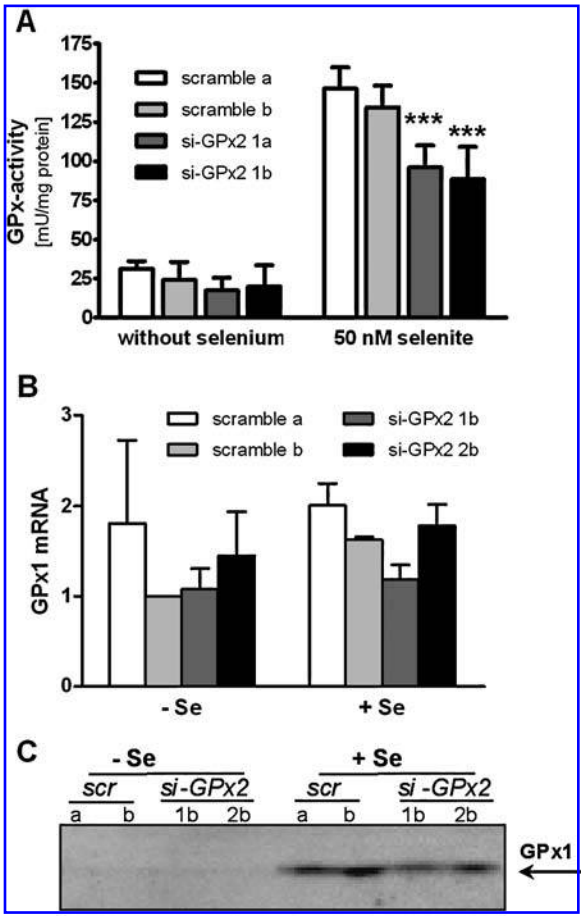

FIG. 7. GPx2 knockdown reduces total GPx activity but does not influence GPx1 expression. (**A**) Total GPx-activity. Cells were cultured as indicated. Lysates were analyzed for GPx activity using H_2O_2 as substrate. $^{***}p < 0.001$ *vs.* scrambles. (**B**) GPx1 mRNA: Cells were grown with or without selenium supplementation. GPx1 cDNA was estimated by qRT-PCR. Values are means ± SD ($n = 2$). (**C**) GPx1 protein: Cells were grown as indicated and GPx1 measured by Western blot analysis ($n = 3$).

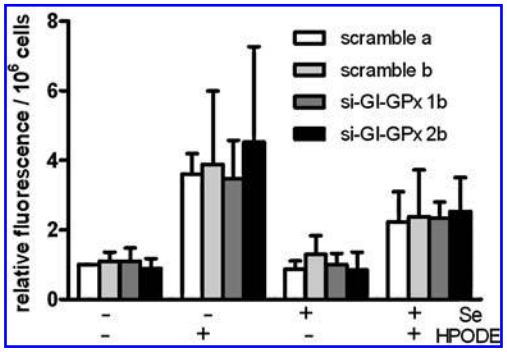

FIG. 8. Hydroperoxide concentration in control and si-GPx2 clones. Cells were grown ± Se for 3 days. After loading with 20 μM DHR123 for 30 min, cells were treated or not with 20 μM HPODE for 15 min. Fluorescence was normalized to unchallenged selenium-deficient scramble a. Values are means ± SD (n = 3). For details, see Methods section.

pression. Thus, COX-2 expression is inversely related to the GPx2 content of HT-29 cells. COX-2 mRNA turnover did not differ in GPx2 knockdown and scramble cells (data not shown). Hence, the high amounts of COX-2 do not result from an increased mRNA stability but are most likely due to an enhanced transcription of the COX-2 gene.

GPx2 also affects COX-2 levels in IL-1-stimulated cells

COX-2 is induced by the pro-inflammatory cytokine IL-1 (37). The expected increase of COX-2 transcripts upon IL-1β treatment, however, was only marginal in scramble cells, whereas basal COX-2 transcripts and protein were high in si-GPx2 clones, as shown before, and significantly increased after IL-1β stimulation (Fig. 4A/B). Again, the selenium status did not influence COX-2 expression (compare white and light gray with dark gray and black columns in Fig. 4). Thus, the regulation of COX-2 expression by GPx2 is even maintained under inflammatory conditions.

GPx2 affects PGE_2-production also by modulation of mPGES-1 expression

In accordance with high COX-2 levels an increased release of PGE_2 was observed in si-GPx2 clones (Fig. 5*A*). Under IL-1-stimulation, however, clone 2b produced markedly more PGE_2 than clone 1b. Since this did not quantitatively reflect the elevated COX-2 levels, an additional interference of GPx2 with the arachidonate cascade was suspected. In fact, the microsomal PGE synthase (mPGES-1), the enzyme producing PGE_2 from the primary COX-2 products PGG_2/PGH_2, was dramatically enhanced by GPx2 knockdown too (Fig. 5B). A comparison of released PGE_2 and mPGES-1 transcript (Fig. 5A/B) reveals an almost ideal correlation. Thus, GPx2 can influence the pathway of PGE_2 production at two levels, by dampening activity/expression of the key enzyme of the cascade, COX-2, and by suppressing mPGES-1.

GPx2 expression is independent of COX-2

In order to find out about a mutual interaction between GPx2 and COX-2, HT-29 cells in which COX-2 expression was stably suppressed by si-RNA were generated. Two clones were selected for further analysis. Both clones showed a markedly suppressed COX-2 expression that nevertheless increased upon IL-1 stimulation (Fig. 6A). However, GPx2 expression was not affected by a COX-2 knockdown. At mRNA and protein level, GPx2 expression in the si-COX-2 clones was comparable to the scramble cells (Fig. 6B/C).

Hence, a knockdown of GPx2 results in a clear increase in COX-2 expression, whereas COX-2 itself does not appear to have an effect on GPx2, indicating that the interplay between GPx2 and COX-2 is only unidirectional.

The GPx2 effect is specific

Since HT-29 cells express both GPx1 and GPx2, GPx activity and GPx1 expression in scramble and si-GPx2 clones was determined. Due to a similar substrate specificity, the activity of GPx1 and GPx2 cannot be estimated individually (5). Therefore, total GPx activity was measured with H_2O_2 as substrate. About 65% of the activity of the scramble clones was retained in GPx2-free si-GPx2 clones 1a and 1b (Fig. 7A), indicating that GPx2 accounts for only one-third of total GPx-activity in HT-29 cells. Residual activity in selenium-deficient si clones might result from residual amounts from GPx1/2 or, more likely, from GPx4. None of the si-GPx2 constructs affected the level of GPx1 mRNA (Fig. 7B) or protein (Fig. 7C). Undetectable GPx1 protein in selenium deficiency together with the obvious lack of an effect of the selenium status on COX-2 expression (see Fig. 3D, scramble) clearly shows that none of the selenoproteins, the translation there from is usually downregulated in selenium deficiency, contributes to COX-2 regulation. Both observations further exclude secondary metabolic changes resulting from selenium deficiency as regulators of COX-2 expression.

Finally, it was investigated whether the regulation of COX-2 is simply a consequence of an altered global hydroperoxide tone in si-GPx2 cells. In unchallenged cells, hydroperoxide levels were indistinguishable in the -Se and +Se state (Fig. 8). Due to the low GPx1 expression, intracellular hydroperoxide concentrations were higher upon treatment with hydroperoxy-octadecadienoic acid (HPODE) in -Se cells. Under none of the conditions a difference between scramble and si-GPx2 clones was observed, which implies that hydroperoxides applied via the cell culture medium are preferentially degraded by GPx1. The results show that GPx2 does not substantially influence the overall cellular hydroperoxide tone but leaves the option that it acts in specific compartments. Taken together, the findings indicate that the enhanced COX-2 expression here observed specifically results from low GPx2.

Discussion

Both GPx2 and COX-2 are upregulated in inflammatory and preneoplastic conditions. As shown here, both enzymes are co-localized, partially at least, in the endoplasmic reticulum of colorectal cancer cells, which led to the hypothesis that they might influence each other in their activity and/or expression. The counteraction of COX-2-mediated PGE_2 production by GPx2 confirms the hypothesis and provides an explanation for the postulated anti-inflammatory action of GPx2.

At first glance, the underlying mechanism appears to be trivial: GPx2 removes hydroperoxides required for COX-2 activity and thereby interrupts the autocrine loop by which

PGE_2 has been shown to induce COX-2 (16, 19), as well as mPGES-1 (27). The relevance of the hydroperoxide level to inflammatory processes in the gastrointestinal system is indeed evident from the spontaneous development of ileocolitis and intestinal cancer in GPx1/2 DKO mice. However, since neither of the single KO strains displayed a comparable phenotype (12, 20), a synergistic anti-inflammatory activity of GPx1 and GPx2 has to be postulated. But the role of GPx2 appears to be more important, since a single allele of *gpx2* but not of *gpx1* proved to be sufficient to prevent inflammation in GPx1/2 DKO mice (15). The findings presented here clearly corroborate this view, since an increased COX-2 expression was never observed in selenium-deficient cells in which GPx1, as opposed to GPx2, was nearly absent. Plausibly, the pivotal importance of GPx2 results from its strategic localization in the endoplasmic reticulum, where it can interfere with COX-2 activity by local removal of hydroperoxides and, thus, regulate the level of PGE_2.

PGE_2 is the common denominator in regulating COX-2 and mPGES-1 expression. It acts by binding to G-protein coupled receptors EP1-4. Transcriptional regulation of COX-2 expression further includes the activation of C/EBP-β, phosphoCREB, c-MYB, AP-1, NFκB, and β-catenin/TCF/LEF, the latter also being activated by PGE_2 (7), as reviewed in Ref. (37). Thus, the effect of GPx2 on COX-2 expression appears to be linked to the Wnt pathway, which often is dysregulated in colon cancer (3) but required, in a regulated way, to maintain intestinal homeostasis (35). The activation of the GPx2 promoter by β-catenin (24) further links GPx2 to Wnt and points to a role in the proliferation of mucosal epithelial cells (see below).

Factors upregulating GPx2 in inflamed and cancer tissue are not known so far. Neither is it known what comes first, upregulation of COX-2 or GPx2. Both enzymes are present in affected tissues (see Fig. 2). In the surgical samples analyzed, the time course of disease development could not be monitored. At best, Fig. 2 shows a tendency towards higher COX-2 expression in early stages of colitis, indicating that expression of COX-2 might precede that of GPx2. In healthy tissue, GPx2 is required to maintain homeostasis of the intestinal epithelium, which is consistent with its localization in the proliferative zone in the crypt grounds (17); its induction via ΔpNp63 (46), a transcription factor characteristic for undifferentiated basal epithelial cells (47); and its downregulation in mice with a mutated NKx3.1, a homeobox gene required for regulation of epithelial differentiation (34). GPx2 can be induced via an Nrf2-responsive element in its promoter (1). Upregulation, therefore, might be part of the adaptive response. Activators of Nrf2 comprise a number of phytochemicals with anti-inflammatory capability that also activated the GPx2 promoter (25) as well as endogenous pro-inflammatory stimuli, which are either produced by COX-2 itself or by COX-2-activated pathways, such as hydroperoxides (26) or the late COX-2 product 15-deoxy-$\Delta^{14,15}PGJ_2$ (22). The importance of Nrf2 in the regulation of GPx2 expression is further corroborated by the significant increase of COX-2 in the colonic mucosa of $Nrf2^{-/-}$ mice (23) in which GPx2 is decreased (39). Any increase of COX-2 may therefore be suspected to favor GPx2 expression. The impossibility to upregulate GPx2 expression by COX-2 suppression (Fig. 6) is in line with this assumption.

In conclusion, the findings presented provide a mechanism for the postulated anti-inflammatory function of GPx2 in the gastrointestinal system. By counteracting COX-2 expression, the GSH/GPx2 couple appears to be the physiological buffering system to prevent undue activation of COX-2 and, thus, exacerbation of inflammation. As a target of Nrf2, which is activated by endogenous inflammatory stimuli, GPx2 may also act as a relay to shut off any inflammatory process to prevent its chronification and, in consequence, inflammation-triggered carcinogenesis.

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Abbreviations

COX-2, cyclooxygenase-2; GPx1, glutathione peroxidase 1; GPx2, gastrointestinal glutathione peroxidase; GSH, glutathione; HPODE, hydroperoxy octadecadienoic acid; IL-1, interleukin 1β; mPGES, microsomal prostaglandin E synthase; Nrf2, NF-E2-related factor 2; PGE_2, prostaglandin E_2.

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