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Nitric Oxide Exposure of CC531 Rat Colon Carcinoma Cells Induces γ-glutamyltransferase which May Counteract Glutathione Depletion and Cell Death

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γ-Glutamyltransferase (GGT) has a central role in glutathione homeostasis by initiating the breakdown of extracellular GSH. We investigated in the present study whether nitric oxide exposure of CC531 rat colon carcinoma cells modulates GGT and how the activity of the enzyme affects the level of intracellular GSH. The data show that GGT activity was induced in a dose-related manner by two NO-donors (spermineNONOate and nitrosoglutathione) and that antioxidants partly inhibited the induction. SpermineNONOate lowered intracellular GSH and induced apoptosis. Cultivating the cells in cystine-depleted medium also resulted in a 50% lowering of GSH, but this was avoided when GSH was added to the medium. This effect was mediated by the activity of GGT and shown after inhibiting GGT activity with acivicin and cyst(e)ine transporters with alanine and homocysteic acid. This shows that the cells benefit from GGT in maintaining the intracellular GSH level. Cells with induced GGT activity obtained after NO incubation showed a higher uptake rate of cysteine (2-fold), measured by incubating the cells with ^{35}S-radiolabeled GSH. The enzyme was also induced by interferon-γ and tumor necrosis factor-α, but this induction was not connected to activation of the endogenous nitric oxide synthase, as the addition of aminoguanidine, a NO-synthase inhibitor, did not affect the induction. The present study shows that the activity of GGT is upregulated by NO-donors and that the colon carcinoma cells, when cultivated in cystine-depleted medium, benefit from the enzyme in maintaining the intracellular level of GSH. Thus, the enzyme will add to the protective measures of the tumor cells during nitrosative stress.

Keywords: γ-glutamyltransferase; Glutathione; Oxidative stress; Nitric oxide; Antioxidant; Colon carcinoma cells

INTRODUCTION

Glutathione is the most abundant intracellular non-protein thiol. This tripeptide is an important antioxidant against free radicals and can be significantly lowered during oxidative stress. Depletion of intracellular GSH is one of the early steps in apoptosis. As most cells have no uptake mechanism of GSH, they depend on resynthesis to prevent cell death. A limiting factor in cellular GSH synthesis is the availability of cyst(e)ine (for recent reviews, see Ref. [1,2]).

The enzyme γ-glutamyltransferase (GGT) initiates the breakdown of extracellular glutathione by hydrolysing the γ-glu-cys bond in GSH. The remaining part, cys-glu, is then hydrolysed by dipeptidases. Cellular uptake of the amino acids will thus provide the monomers needed for GSH biosynthesis. GGT has, therefore, a role in cellular GSH homeostasis and may be of significant importance when the level of cyst(e)ine is reduced.[3–5]

Fibroblasts requiring cysteine for growth were able to grow in cysteine-free medium if supplemented with GSH.[3,6] These studies showed that GGT acts as a glutathionase and can provide cells with cysteine. Studies of tumors *in vivo* have shown that they can extract GSH from the circulation and that GGT participates in this

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process.[7,8] The enzyme may thus provide tumors with a growth advantage and play a role in resistance towards oxidative stress. Cells cultivated *in vitro* have access to surplus amounts of cystine (200 μM) when compared to *in vivo* concentrations (5–10 μM). It was recently shown that incubation of lymphocytes in medium devoid of cystine resulted in oxidative stress and apoptosis, but lymphocytes transfected with GGT were protected if GSH was added to the medium.[4] This study confirmed that GGT activity results in an increased uptake of cysteine obtained by degradation of extracellular GSH and in this way protects the cells from GSH depletion and oxidation-induced cell death.

As tumors progress they tend to increase their resistance towards oxidative and nitrosative stress. NO is highly tumoricidal in the presence of H_2O_2[9,10] and these agents appear to be part of a natural defence towards metastatic growth.[11] It has been shown that GSH protects tumor cells against oxidative stress in liver microvasculature[12] and that tumor cells with elevated GSH or ability to resynthesise GSH possess a higher invasive potential.[10,13] GSH also plays an important role in the protection of tumor cells against NO-mediated apoptosis.[14,15] Intracellular GSH will, therefore, contribute to the mechanisms of tumor cell survival during metastatic growth. Furthermore, oxidative mechanisms are implicated in the cytotoxic effect of various anti-cancer drugs and the higher resistance towards oxidative stress may, therefore, be of importance in the development of drug resistance.

We and other investigators have shown that GGT is induced after exposure of cancer cells to peroxides.[16–19] When this is linked to the ability of the enzyme to provide cyst(e)ine, it appears that GGT has an important role in GSH salvaging during oxidative stress. Our aim was to investigate whether the enzyme is also induced by nitrosative stress, such as that produced by incubating tumor cells with NO-donors or after induction of the endogenous nitric oxide synthase. We then set out to find whether GGT helps these tumor cells to avoid apoptosis in situations with depleted cysteine, by degrading GSH in the medium. We also investigated whether cells with induced GGT have an increased ability to obtain cysteine from such degradation. NO is a pleiotropic agent, and has been reported to deplete cells of GSH at higher concentrations resulting in cell death and apoptosis. We found that GGT was induced by NO and restricted cell death when incubating the tumor cells in cyst(e)ine reduced medium.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

CC531 is a colon carcinoma cell line that was originally developed in rats after chemical carcinogenesis.[20] The cells were cultured in RPMI 1640 medium with 5% fetal calf serum in a humid atmosphere with 5% CO_2 at 37°C. The cells were replated by trypsinization each 4–6th day and tested regularly for mycoplasma (Mycoplasma PCR Elisa, Roche Diagnostics, Basel, Switzerland).

Treatment of Cells for Enzyme Induction

Viable cells (0.5×10^6) were seeded in 25 cm^2 flasks with 5 ml medium, or in 6 well plates (0.2×10^6 cells per well) with 3 ml medium and allowed to proliferate overnight. Then spermineNONOate (SpNO) or nitrosoglutathione (GS-NO) (both purchased from Calbiochem, Merck Eurolab, Darmstadt, Germany) was added to the medium for a period of 4 h. After change to normal growth medium the cells were further incubated for 48 h before harvesting. To ensure rapid degradation of GS-NO by GGT the co-substrate glycylglycine was added (final concentration of 25 mM). Three antioxidants were added to the medium during the first 24 h incubation periods with SpNO; 10 mM *N*-acetylcysteine (NAC, Sigma Aldrich), 500 mU/ml catalase (Sigma Aldrich, St.Louis, Mo) and 1 mM ebselen (Calbiochem). Cells were also incubated with a mixture of murine recombinant cytokines (interferon and tumor necrosis factor (IFN-γ and TNF-α, R&D Systems, UK) for 24 h (final concentrations 100 U/ml and 1.5 ng/ml, respectively.), and then in normal growth medium for 24 h before being harvested. To inhibit the inducible nitric oxide synthase, aminoguanidine (Sigma-Aldrich) was added (final concentration 1 mM).

Cell Harvesting and Enzyme Measurements

Cells were harvested by trypsinization, washed in normal growth medium and counted. The cells were then solubilized at a concentration of 2×10^6 cells/ml in phosphate buffered saline (PBS) with 1% Triton X-100 by gentle mixing for 30 min at room temperature. The supernatant after a brief centrifugation (5 min at 5000*g*) was collected for GGT activity measurements. To estimate the amount of dead or dying cells floating in the medium after SpNO incubations, the medium was collected, centrifuged and the pellet was solubilized in 250 μl PBS with 1% Triton X-100 before measurement of lactate dehydrogenase (LDH). GGT and LDH activities were determined at 37°C according to the recommendations of the International Federation of

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Clinical Chemistry (IFCC), using a centrifugal analyzer (Cobas Fara, Roche Diagnostics, Basel, Switzerland) with commercial kits (γ-GT and LDH optimized kits, Boehringer Mannheim Lab Diagnostics, Germany).

RNA Isolation and GGT mRNA Quantitation

Total RNA was isolated from cultured cells using Trizol reagent (Gibco BRL, Life Technology, NY, USA), according to the recommendations from the manufacturer. For RT-PCR, 1 μg of RNA was used together with RNAsine (Promega, Madison, WI, USA), oligodT (Gibco BRL) and Superscript TM reverse transcriptase (Gibco BRL). For PCR, the cDNA was amplified with Dynazyme (Finnzyme Oy, Espo, Finland) and gene specific primers for GGT mRNA[21] and GAPDH using the primer-drop method.[22] The PCR products were visualized by ethidium bromide staining after electrophoresis, and the gels were scanned using BioRad Multianalyst TM/PC (BioRad Labs, Oslo, Norway).

Western Blots

Cells ($4-5 \times 10^6$) were harvested and sonicated in 200 μl PBS. Protein concentration was measured using BioRad DC protein assay (Bio-Rad Labs) with bovine serum albumin as standard. After dilution to 0.5 mg/ml of total protein, a volume of 10 μl was mixed with 5 μl SDS sample buffer (0.125 M Tris–HCl pH 6.8, 8% SDS, 5% mercaptoethanol) and 5 μl tris-buffered saline. After denaturation (boiling for 5 min) the samples were run on a precast 20% SDS-polyacrylamide gel (Homogeneous 20, Amersham Pharmacia Biotech, Uppsala, Sweden) in the PhastSystem which was also used for blotting onto PVDF membranes (Amersham Pharmacia). Protein bands were detected using the Chemiluminescence Western Blotting kit (Roche-Diagnostics). The primary antibody was a polyclonal anti-rat-kidney GGT (IgG-fraction) purified from rabbit.[23]

Measurement of Nitrite

The amount of NO_2^- was measured with the Griess reaction. Medium (50 μl) obtained after incubations of cells with SpNo were mixed with 50 μl of a freshly prepared 1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% naphtylenediamine–HCl in water. Absorbance was measured at 550 nm in a microplate reader (Titertek Multiskan PLUS, Labsystems, Finland).

Measurement of GSH

Cells (0.75×10^6) were seeded in 6 cm plates and incubated overnight in normal growth medium. After exposing the cells of SpNO or incubating them in cystine-depleted medium, the cells were washed with cold PBS and harvested by scraping in 300 μl metaphosphoric acid (5%, w/v). The supernatant obtained after centrifugation at 12000*g* for 10 min at 4°C was analysed using the GSH assay kit (Calbiochem) which is based on the non-enzymatic reaction of thiols with a quinolinium chromogen. Crystalline GSH (Sigma–Aldrich) was used as standard. The precipitated proteins were solubilized in a volume of 300 μl 0.1 M NaOH and quantified. The GSH content was expressed as nmol GSH/mg protein.

Apoptosis and DNA Fragmentation Assays

Detection of apoptosis after SpNO exposure, was obtained using the DNA laddering technique. Cell pellets were obtained either by trypsinization of viable cells still attached to the plastic culture flasks, or by centrifugation of growth medium to obtain unattached, floating cells. The cells were washed twice in PBS and incubated in 50 μM Tris–HCl pH 8.0, with 10 mM EDTA, 0.5% SDS and 0.5 mg/ml proteinase K for 3 h at 50°C. After adding proteinase K (0.5 mg/ml) the incubation was continued for one more hour. DNA was separated in a 1.4% agarose gel and visualized by UV illumination after ethidium bromide staining. Quantification of apoptosis was determined using the DNA Fragmentation ELISA kit (Boehringer Mannheim), according to the procedure recommended by the manufacturer. In brief, cells were seeded in 6-well plates (2×10^5 cells/well) and incubated for 24 h in control medium with 1 μM BrdU, then in cystine-depleted medium (with or without 25 μM GSH) for 24 h before being harvested for apoptosis assay. The final measurement was performed after stopping the ELISA reaction using H_2SO_4 and reading the absorbance at 450 nm. Data from experimental cells were shown relative to those of control cells.

Uptake of Radiolabeled Cysteine

Cells, either control cells or cells that had been incubated 96 h earlier for a period of 4 h with 200 μM SpNO, were seeded in 6-well plates (2×10^5 cells/well) with 3 ml normal growth medium. After 24 h, the medium was changed to cystine-depleted medium to which was added ^{35}S-labelled GSH (purchased from Perkin–Elmer Life Sciences, Boston, USA) at 0.5 μCi/ml, 25μM GSH and 25 mM glycylglycine. A series of control cells were also incubated in this medium with 500 μM acivicin. After incubation periods of 15 min, 2, 4 and 8 h, the cells were washed twice with 1 ml PBS and solubilized in 1.0 ml 0.1 M NaOH with 0.1% SDS at 37°C. Radioactivity was counted after mixing 250 μl

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of cell lysate with 3.0 ml Ultima Gold (Packard Instruments, CT, USA) in a Packard 1900 TR liquid scintillation counter.

Statistics

Statistical data were obtained using the SAS STAT software (SAS Institute Inc., Cary, NC, USA). Comparison of mean values between two groups was performed using Student's *t*-test and for multiple comparisons (ANOVA) the Dunnett option was used. Differences with *p*-values less than 0.05 were considered significant.

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RESULTS

GGT is Induced by NO-donors

Incubations of CC531 cells with SpNO resulted in a dose-dependent increase of GGT activity (Fig. 1**A**). A three-fold increase in GGT activity was detected after incubating the cells for 4 h with 200 μM SpNO followed by 2 days incubation in normal growth medium. This increase was partly, but not totally blocked by addition of the antioxidants ebselen, *N*-acetylcystein and catalase (Fig. 1**A**). Increased amount of GGT protein was detected 48 h after the SpNO incubation in Western blot analysis (Fig. 2**A**), whereas the amount of GGT mRNA was slightly but not significantly altered when analysed 6–24 h after the SpNO exposure (Fig. 2**B**). GGT was also induced by the NO-donor GS-NO with the addition of 25 mM glycylglycine. After 4 h incubations of the cells with 1 mM GS-NO, the increase in GGT was 2-fold when measured 48 h later (Fig. 1**B**). The generation of NO was monitored by measuring the concentration of NO_2^- in the medium. NO_2^- was maximally elevated after 2 h of SpNO incubation and 4 h of GS-NO incubation, the range being 80–120 μM with 200 μM SpNo and 1 mM GS-NO.

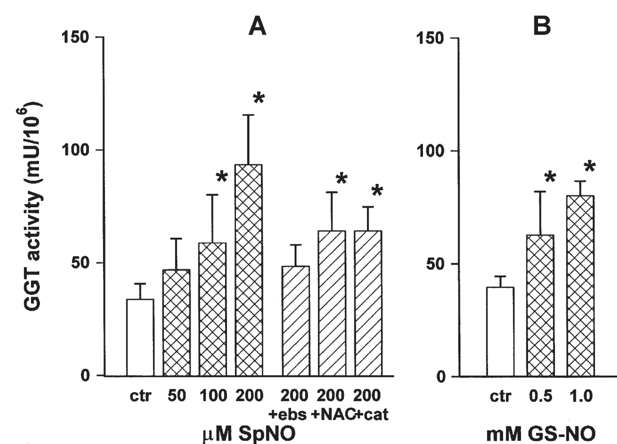

FIGURE 1 *Induction of GGT after incubation of CC531 cells with* ***A*** *SpNO and* ***B*** *GS-NO.* GGT activity was measured 48 h after 4-h incubation period of the cells with SpNO **A** or GS-NO **B** in the medium. Incubations with antioxidants (1 mM ebselen (ebs), 10 mM *N*-acetylcysteine (NAC), or 500 mU/ml catalase (cat)) were performed together with 200 μM SpNO. Data shown are means (+ SD) of 4–6 experiments. Data sets were compared using ANOVA and *indicates a significant difference in activity ($p < 0.05$) compared to that of control cells.

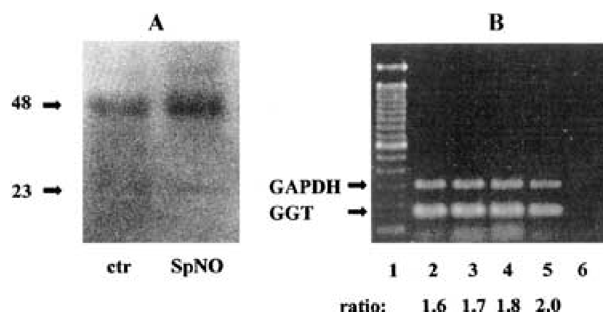

FIGURE 2 *Western blot and quantitative PCR.* **A**: Western blot of extracts from CC531 cells incubated in standard growth medium only (ctr), or from cells exposed to 200 μM SpNO for 4 h and then incubated for 48 h in normal medium before harvesting (SpNO). After blotting, bands were visualised using a primary antibody against rat kidney GGT and a chemiluminescence Western blot kit. Upper arrow indicates the large GGT subunit (M_r 48 kD), the lower arrow the small subunit (M_r 23 kD). **B**: Quantitative PCR of GGT mRNA and GAPDH mRNA using the primer drop method. Lane 1: DNA ladder, lane 2: control cells, lanes 3–5: cells incubated with 200 μM SpNO for 4 h and harvested either 6, 12 or 24 h later, respectively. Lane 6: negative control. After densitometric scanning of the bands, their relative intensities (ratio GGT/GAPDH) were estimated as shown in the figure.

The Effect of SpNO on Cell Proliferation and Death

The number of viable cells was found to be significantly decreased after incubation with 200 μM SpNO, as detected by counting the viable cells 48 h after exposure (Fig. 3**A**). At this time, an increasing number of dead or dying cells was detected floating in the medium. A quantitative estimate of these cells was obtained after harvesting the floating cells and measuring the LDH activity after lysis (Fig. 3**B**). Agarose gel electrophoresis of DNA extracted from these floating cells demonstrated the apoptotic DNA ladder pattern (Fig. 3**C**).

Response of Intracellular GSH to NO Exposure

The intracellular GSH level was significantly decreased after 4 h of SpNO incubation. Continued incubations of the cells in normal growth medium for another 20 h, resulted in increased GSH of the cells exposed to SpNO, about 80% higher than the control level at 24 h (Fig. 4).

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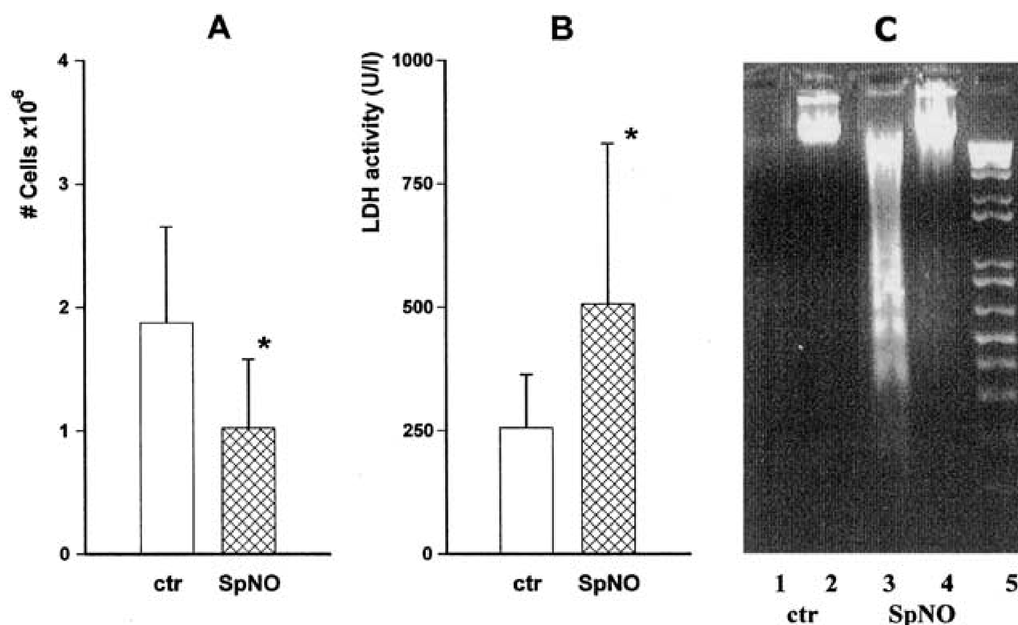

FIGURE 3 *Cell viability after incubation of CC531 cells with SpNO*. Cells were seeded in 25 cm^2 flasks (0.5 × 10^6/flask), incubated with 200 μM SpNO in the medium for 4 h, and then in normal growth medium for 44 h. **A**: The number of viable cells in culture 48 h after SpNO exposure was counted (SpNO) and shown together with control cells (ctr) grown in normal growth medium only. **B**: The activity of LDH was determined in cells floating in the medium after being harvested by centrifugation and lysed in Triton X-100. Data shown are means (+SD) of 4–8 measurements. **C**: Detection of apoptosis by DNA ladder pattern. Control cells (ctr) and cells treated with SpNO were harvested either from the culture medium (lanes 1 and 3) or detached from culture flasks by trypsinization (lanes 2 and 4). After incubations with proteinase K and ribonuclease A, the DNA was separated by agarose electrophoresis. Lane 5: DNA ladder standard. The SpNO data sets were compared to control cells using Student's *t*-tests, and *indicates a significant difference.

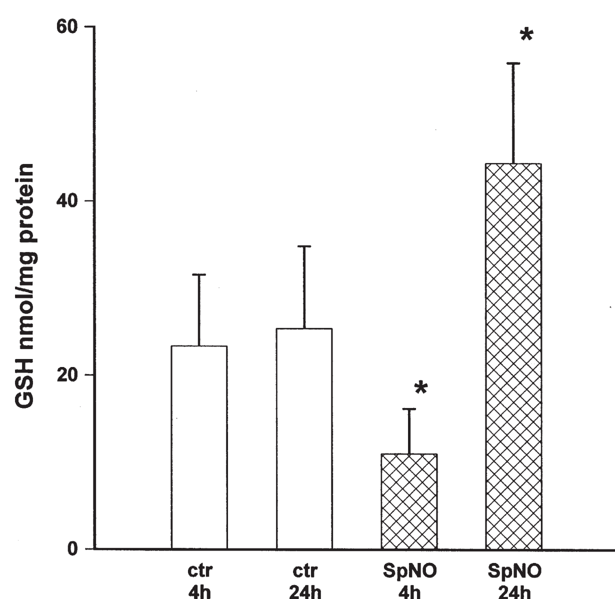

FIGURE 4 *Intracellular GSH levels after NO-exposure*. Cells were incubated 4 h with SpNO (200 μM) followed by 20 h in normal growth medium. The amount of GSH was determined in cell lysates after the 4 h SpNO exposure, or 20 h later. Control cells (ctr) were incubated in normal growth medium only. Data are from 3–5 experiments, and shown as mean + SD. The GSH level after SpNO treatments were compared to the respective controls using Student's *t*-tests and *indicates a significant difference ($p < 0.05$).

Induction of GGT by IFN-γ and TNF-α

Induction of GGT was also noted after incubations with interferon-γ and tumor necrosis factor-α (Fig. 5). The addition of 1 mM aminoguanidine did not prevent the induction (Fig. 5), indicating that the endogenous nitric oxide synthase is not part of this induction. Furthermore, we were unable to detect any NO_2^- being produced during these incubations. Incubation of either of the cytokines alone also induced GGT, but to a lower degree. Higher concentrations of the cytokines were not tried as apoptosis was induced.

GGT Prevents GSH Depletion and Cell Death in Cystine Depleted Medium

The effect of GGT as a supplier of cysteine was studied in cystine-depleted medium to which GSH was added. Incubating cells in cystine-depleted medium resulted in a strong reduction in intracellular GSH (Fig. 6), which was prevented by adding GSH (50 μM) to the medium. Inhibition of GGT activity with acivicin, which diminished the activity by more than 90%, or the cyst(e)ine transporters by homocysteic acid and alanine, resulted in lowered GSH in spite of the added extracellular GSH. Incubation of cells in cystine-depleted medium also resulted in a significant increase in apoptotic cells as quantified by the cellular DNA fragmentation

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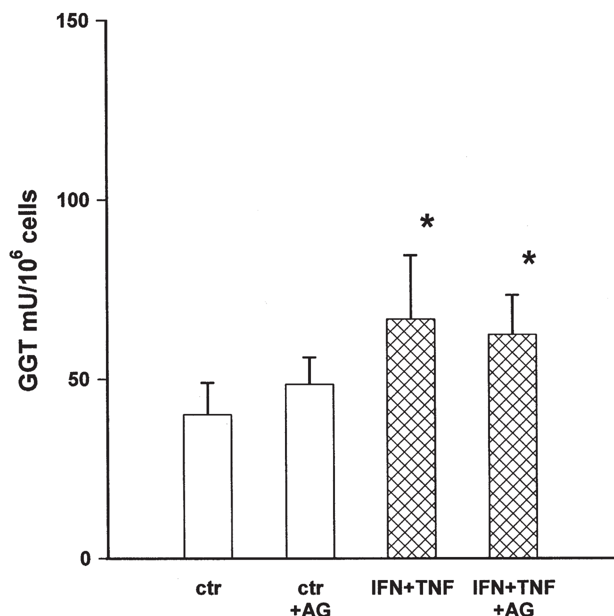

FIGURE 5 *Induction of GGT after incubation with cytokines*. GGT activity was measured after a 24 h incubation of the cells with IFN-γ (100 U/ml) and TNF-α (1.5 ng/ml) followed by 24 h in normal growth medium without cytokines. AG indicates aminoguanidine that was added to the medium together with the cytokines. Control cells were incubated in normal growth medium for 48 h without cytokines. Data shown are means (+SD) of 4–6 experiments. *indicates a significant difference in activity ($p < 0.05$) using Student's t-tests compared to that of respective control cells.

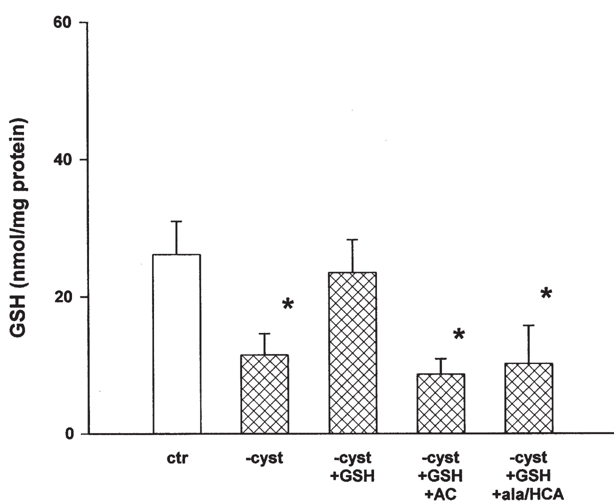

FIGURE 6 *Intracellular GSH after incubation in cystine-depleted medium*. GSH was measured in the CC531 cells after 24-h incubation in control medium (ctr), in cystine-depleted medium (−cys), or in this medium with 50 μM GSH (−cys/ + GSH), with GSH and acivicin (−cys/ + GSH + AC), or with GSH and alanine/homocysteic acid (−cys/ + GSH/ + ala/HCA). Control cells (ctr) were incubated in normal growth medium only. Data are from 3–6 experiments and shown as mean +SD. *indicates a significant difference ($p < 0.05$) to GSH levels in control cells using ANOVA.

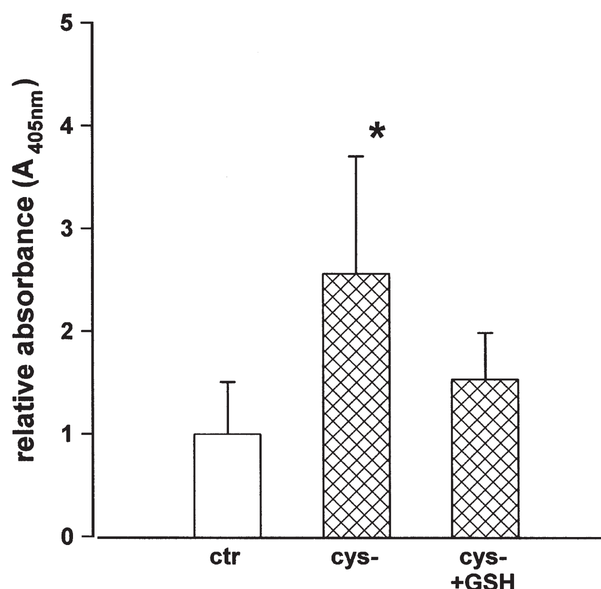

FIGURE 7 *Apoptosis in CC531 cells after incubation in cystine-depleted medium*. Cells were incubated in cystine-depleted medium (cys −) and in cystine-depleted medium enriched with GSH (50 μM) for 24 h, and the amount of apoptotic cells was quantified using the DNA fragmentation ELISA kit. Control cells were incubated in normal growth medium. Data shown are means (+SD) from 3–4 experiments. *indicates a significant difference ($p < 0.05$) from cells in standard medium using ANOVA.

ELISA. This number was significantly reduced when GSH was added to the medium (Fig. 7).

Cells Preincubated with SpNO Show an Increased Uptake Rate of Cysteine

To test whether cells with induced GGT had an increased availability of cysteine, we measured the uptake of cysteine. This was performed in cystine-depleted medium, to which was added ^{35}S-radiolabeled GSH. The uptake was almost linear during 8 h and was blocked by inhibiting GGT with acivicin (Fig. 8). The cysteine uptake was almost doubled in cells that had been preincubated 4 days previously with a 4 h exposure to 200 μM SpNO (Fig. 8), the GGT activity level of these cells was about twice the control level.

DISCUSSION

In the present study, we have shown that the activity of GGT in colon carcinoma cells is induced after exposure to NO and that the cells, when cultivated in cystine-depleted medium, benefit from the enzyme in maintaining the intracellular level of GSH. Furthermore, cells with upregulated GGT have a higher rate of cysteine uptake, which results from increased degradation of extracellular GSH. The GGT activity will thus add to the protective measures of tumor cells during nitrosative and oxidative stress

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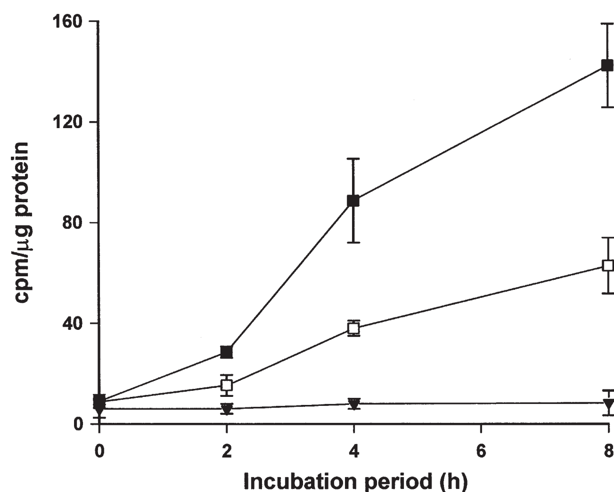

FIGURE 8 *Cysteine uptake during incubation with radiolabeled GSH.* CC531 cells were seeded in 6-well plates (2×10^5 cells/well), grown in standard growth medium for 24 h and then incubated with ^{35}S-GSH and glycylglycine in cystine-depleted medium. The cells were harvested NaOH-SDS and the radioactivity were counted in a scintillation counter. The cells were either control cells (– ~ –), control cells with acivicin (–□–), or cells with induced levels of GGT (–□–). These latter were obtained by incubating cells with 200 µM SpNO for 4 h, and then in normal growth medium for 4 days before cysteine uptake measurements. The data are means of 3 experiments, each with 3–4 parallels.

particularly at low extracellular concentrations of cysteine.

The two NO-donors used, SpNO and GS-NO, upregulated the activity of the enzyme in a dose-related manner. The enzyme was also induced by a third NO-donor (DEA-NO) in a similar manner (data not shown). The SpNO exposure resulted in an increased amount of GGT protein, as revealed by Western bolts, but not in increased total GGT mRNA which may indicate a translational regulation of the enzyme during nitrosative stress.

The different NO-donors affected the induction of the enzyme somewhat differently, which may be related to their different rates of NO release and to distinct effects on the intracellular GSH level observed after exposure (data not shown). GS-NO is a substrate for GGT; degradation of this compound, which is increased in the presence of the transpeptidase substrate glycylglycine, results in NO release.[24,25] These data therefore indicate that GGT is induced by its own substrate. It has been suggested that locally, high and relevant concentrations of NO may be produced during inflammatory responses,[25] indicating that GGT induction may have a physiological relevance which needs to be investigated.

NO adds to the rather long series of oxidative agents that upregulate this enzyme. We and others have earlier reported that GGT is induced by oxidative stress, such as exposure of hydrogen peroxide and quinones to cells, in a way that partly reflects the present results.[17–19,26,27] These inductions were also blocked by the addition of antioxidants such as *N*-acetylcysteine, GSH-ester or catalase.

GGT acts as a glutathionase under *in vivo* like conditions, that is at low concentration of cyst(e)ine.[3,4,26] The enzyme initiates the degradation of extracellular GSH and the level of GGT activity has been shown to correlate to the rate of cysteine uptake.[3,4,6,16,26] GSH is of significant importance in cellular defence against oxidative and nitrosative stress and different cell types use different pathways to maintain the intracellular GSH level.[15,28] Data indicate that the overall capability of cells to maintain a critical amount of GSH determines the susceptibility to NO-induced cell death.[28] Acute exposures of cells to NO frequently result in transient depletion of GSH, followed by GSH elevations.[14,29,30] This may be part of the adaptive response reported for endothelial cells after NO exposure; elevated GSH synthesis was found to coincide with the induction of γ-glutamylcysteine synthetase and increased uptake of cystine through the x_c^- amino acid transport system.[30–32] In a recent study on human T_h lymphocytes it was shown that intracellular GSH regulates NO-activated apoptosis, and that the high GGT level in the T_h2 subset specifically protects these cells.[33] This study is thus supported by the present investigation.

The mechanism behind the NO-mediated induction of GGT remains to be evaluated, but free radicals produced during either oxidative stress or nitrosative stress may be the inducing agents. In support for this are the effects of antioxidants which in part blocked the induction and our earlier findings that GSH depletion alone (using buthionine sulfoximine, BSO and diethyl maleate, DEM) did not induce GGT.[18,19] During such situations, reactive oxygen species may be generated, but apparently not sufficient to induce GGT in the CC531 cells. We also found that incubation of the CC531 cells in cystine-depleted medium, which results in depletion of intracellular GSH, did not induce GGT (data not shown).

In the present study, we also tested whether GGT induction may be mediated through the endogenous NO synthase, but observed that although GGT was induced after incubating the cells with IFN-γ and TNF-α, this was not through induction of iNOS. We also showed that the amount of NO_2^- being produced was low, indicating that the endogenous production of NO was insufficient for GGT induction and that diverse mechanisms are responsible for GGT induction. These data appear to contrast a study on Sertoli cells where GGT induction was reported after incubation of the cells with NO-donors and also after IL1β incubations. It was concluded that the GGT induction was caused by activation of NFκB and iNOS with a subsequent

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cellular production of NO.[34] Although it is known that TNF-α modulates GGT activity[35,36] the mechanisms behind these regulations remain unknown.

The genetic regulation of GGT is complex. In rats, the enzyme is coded for by one gene, but 7 mRNA isotypes have been described. They have identical coding regions but differ in the 5′- untranslated regions. The presence of several promoters and regulatory elements strongly indicates that the enzyme is subjected to a strict regulation (for a recent review, see Ref. [37]. They may also explain how several mechanisms can upregulate GGT during oxidative stress, drug exposure or butyrate mediated differentiation.[16–19,26,38]

The colon carcinoma cell line CC531, established after chemical carcinogenesis in rat (Marquet-84), has a relatively high GGT activity.[18] We found that GGT can supply the cells with cyst(e)ine by hydrolyzing extracellular GSH when the cells were incubated in medium with low cystine, and thus confirmed data from transfected lymphocytes.[4] GGT can in this way add to the protection of tumor cells against oxidative and nitrosative stress.

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