GLUTATHIONE SUPPLEMENTATION POTENTIATES HYPOXIC APOPTOSIS BY S-GLUTATHIONYLATION OF p65-NFκB

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In murine embryonic fibroblasts, N-acetyl-L-cysteine (NAC), a GSH generating agent, enhances hypoxic apoptosis by blocking the NFκB survival pathway (J. Biol. Chem. 279, 50455, 2004). Here, we examined sulfhydryl modifications of the p65 subunit of NFκB that are responsible for NFκB inactivation. In MIA PaCa-2 pancreatic cancer cells, hypoxia increased p65-NFκB DNA binding and NFκB transactivation by 2.6- and 2.8-fold, respectively. NAC blocked events without having an effect on p65-NFκB protein levels and p65-NFκB nuclear translocation during hypoxia. Pharmacological inhibition of the NFκB pathway also induced hypoxic apoptosis, indicating that NFκB signaling pathway is major protective mechanism against hypoxic apoptosis. In cell lysates after hypoxia and treatment with N-ethylmaleimide (NEM, thiol alkylating agent), dithiothreitol (DTT, disulfide reducing agent) was not able to increase binding of p65-NFκB to DNA, suggesting that most sulfhydryls in p65-NFκB protein were in reduced and activated form after hypoxia, thereby being blocked by NEM. In contrast, with hypoxic cells that were also treated with NAC, DTT increased p65-NFκB DNA binding. Glutaredoxin (GRx), which specifically catalyzes reduction of protein-SSG mixed disulfides, reversed inhibition of p65-NFκB DNA binding in extracts from cells treated with hypoxia plus NAC and restored NFκB activity. This finding indicated that p65-NFκB-SSG was formed in situ under hypoxia plus NAC conditions. In cells, knock-down of endogenous GRx1, which also promotes protein glutathionylation under hypoxic radical generating conditions, prevented NAC-induced NFκB inactivation and hypoxic apoptosis. The results indicate that GRx-dependent S-glutathionylation of p65-NFκB is most likely responsible for NAC-mediated NFκB inactivation and enhanced hypoxic apoptosis.

Tumor hypoxia is strongly associated with tumor propagation, malignant progression, and resistance to chemo- and radiation therapy (1). NFκB1 is a redox-regulated transcription factor that is activated during hypoxia (2;3). NFκB belongs to the Rel family, which includes five mammalian Rel/NFκB proteins: RelA (p65), c-Rel, RelB, NFκB1 (p50/p105) and NFκB2 (p52/p100) (4). The inactive form of NFκB is localized in the cytoplasm as p65:p50 (the most abundant form) or p50:c-Rel heterodimers through interaction with IκB repressor proteins (IκBα, IκBβ, IκBγ and IκBε) (5). Once activated, NFκB translocates to the nucleus, where it binds to DNA and activates various target genes including Bcl-xL, Bcl-2, a hematopoietic-specific Bcl-2 homologue A1, caspase-8-FADD-like IL-1β-converting enzyme inhibitory protein, TNFR-associated factor 1 and 2, cellular inhibitors of apoptosis, and X chromosome-linked inhibitor of apoptosis (XIAP/hILP) (6;7).

NFκB family proteins have a conserved domain of ~300 amino acids in the amino terminal region known as the Rel homology region (RHR). RHR consists of a DNA binding domain, a dimerization domain, and a nuclear localization signal (8). The carboxyl terminal domains of NFκB proteins are divided into two classes depending on the presence (p65, RelB and c-Rel) or absence (p50 and p52) of the
transactivation domain (TAD) (9). NFκB has cysteine residues close to or within its DNA-binding loops (9), e.g., Cys 59 of p50 and Cys 38 of p65, that contact the DNA backbone (10). Cys 62 of p50 is required for DNA binding activity (11). Previously, changes in cellular redox state and disulfiram (thiocarbamate) treatment were shown to induce S-glutathionylation and subsequent inactivation of the p50 subunit of NFκB (11;12). Since only p65-NFκB is responsible for transcriptional activation (13), it follows that glutathionylation of the transcriptionally active subunit p65 should have a more profound effect on NFκB activation than glutathionylation of the p50 subunit.

The GSH/GSSG equilibrium is the major redox buffer in cells. Apart from providing cells with a reducing environment ([GSH] >> [GSSG]) and maintaining proteins in a reduced state, the glutathione redox couple dynamically regulates protein function via reversible disulfide bond formation (14;15). Many modifications of protein sulfhydryls occur during oxidative stress, including formation of sulfenic, sulfinic, and sulfonic acids (16). However, S-glutathionylation (i.e., formation of mixed disulfides between protein thiols and GSH (protein-SSG)) is likely the most prominent sulfhydryl modification (11;17). Moreover, reversible protein-SSG formation has also gained prominence as a mechanism of cellular regulation in redox-activated signal transduction (17). Consistent with a regulatory role, S-glutathionylation inactivates some proteins, e.g., glyceraldehyde-3-phosphate dehydrogenase (GAPDH), protein tyrosine phosphatase (PTP1B), nuclear factor-1; while activating others like human immunodeficiency virus-1 protease, microsomal glutathione-S-transferase, and hRas (17).

An oxidative environment with high GSSG can promote protein-SSG formation. However, protein-SSG formation typically occurs intracellularly without substantial changes in the GSH/GSSG ratio, indicating alternative mechanisms of protein-SSG formation that are incompletely understood (18;19). The enzyme glutaredoxin (GRx), also called thioltransferase, reverses protein glutathionylation in a specific and efficient manner. Accordingly, GRx is used to characterize oxidant-induced protein modification as protein-SSG (17). Although GRx is normally regarded as a deglutathionylating enzyme, GRx also catalyzes the reverse reaction, namely GSSG-dependent protein-SSG formation, and remarkably GRx can also enhance the rate of S-glutathionylation of proteins, e.g., actin, GAPDH, and PTP1B, by an alternative mechanism in the presence of glutathione thiol radical (GS'), despite high GSH content (20). The p50 subunit of NFκB is reported to be inactivated by S-glutathionylation, but thus far no report has described an oxidative modification of the p65 subunit.

Here we report that glutathione supplementation with N-acetyl-L-cysteine (NAC) decreases resistance to hypoxia in pancreatic cancer cells by inhibition of p65-NFκB binding to DNA. The inhibition of NFκB is reversible by GRx1 in vitro, and the inhibition is diminished in cells where GRx1 is knocked down, both characteristics indicative of S-glutathionylation of p65-NFκB as the mechanism of inhibition. Thus, S-glutathionylation and inactivation of NFκB leading to hypoxic cancer cell death may provide a useful adjunct to chemotherapy against hypoxic tumors.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents.** Monoclonal anti-NFκB p65, anti-actin, anti-YY-1 and horseradish peroxidase (HRP)-linked antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, MA). Monoclonal antibody against PARP was purchased from Trevigen (Gaithersburg, MD). Propidium iodide and Alexa 488-conjugated goat anti-mouse IgG were from Molecular Probes (Eugene, OR), DRAQ-5 from Alexis Biochemicals, (San Diego, CA), and JSH-23 from Calbiochem (La Jolla, CA). The luciferase reporter construct pNFκB-Luc was from Stratagene (La Jolla, CA). N-acetyl-D-cysteine (D-NAC) was a gift from Dr. Ian Cotgreave (AstraZeneca, Stockholm, Sweden). Recombinant GRx was isolated and purified as described previously (21). NADPH was from Roche Molecular Biochemicals (Pleasanton,
CA). $^{[35}S]$ Glutathione was from ICN Radiochemicals (San Diego, CA). GSH, GSSG reductase (yeast), mono-carboxymethyl-BSA (BSA-CM) and all other chemicals were purchased from Sigma (St. Louis, MO).

**Cell Culture.** Human MIA PaCa-2 pancreatic carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in complete culture medium containing Dulbecco’s Modified Eagle’s Medium supplemented with 25 mM glucose, 10% fetal bovine serum and antibiotics (50 units/ml of penicillin and 50 μg/ml of streptomycin) in a humidified atmosphere of 5% CO$_2$ and 95% air. Cells were grown to a 60-70% confluence before treatments.

**Generation of the GRx1 knock-down cell line.** Phoenix amphotropic cells were transfected with a puromycin-selectable pSUPER.retro.puro retroviral vector (OligoEngine, Seattle, WA) containing specific short hairpin oligonucleotide sequence against GRx1 (GRx1-shRNA) or non-silencing sequence (ns-shRNA) using LipofectAMINE PLUS (Invitrogen, Carlsbad, CA). Viral supernatants were collected and used to infect MIA PaCa-2 cells. Cells were selected and grown in puromycin (0.5 μg/ml) containing medium and screened for GRx1 protein expression and activity.

**Hypoxia Experiments.** MIA PaCa-2 cells were cultured on 60-mm Petri dishes (5 x 10$^5$ cells/dish) for 42 h prior to exposure to hypoxia. Subsequently, cells were preincubated in the presence and absence of NAC for 3 h in complete culture medium supplemented with 10 mM glucose and 25 mM HEPES buffer. Petri dishes were placed in hypoxic Plexiglass chambers (Billups-Rottenberg, Del Mar, CA) and sparged with 95% N$_2$/5% CO$_2$, 0.5% O$_2$/94.5% N$_2$/5% CO$_2$ or 1% O$_2$/94% N$_2$/5% CO$_2$ at 2 psi for 4 min. Chambers were kept at 37°C throughout the course of experiment. The oxygen concentration of the medium inside chambers was monitored continuously for 24 h using a FOXY-18G Fiber Optic Oxygen Sensor (Ocean Optics Inc., Dunedin, FL). After an initial equilibration of oxygen between gas phase and the medium, medium oxygen concentration inside the chamber remained constant during >24 h, indicating no oxygen back diffusion into the chambers. Nevertheless, some residual oxygen did remain inside the chambers (see results).

**Transfection and Reporter Assay for NFκB Activity.** MIA PaCa-2 cells (5 x 10$^5$ cells/dish) were plated on 60-mm Petri dishes for 30 h. Subsequently, cells were transiently transfected with 2 μg of plasmid containing 5X NFκB promoter fused to the firefly luciferase gene (Stratagene, La Jolla, CA) or 2 μg of empty vector luciferase construct using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). To normalize the transfection efficiency, cells were co-transfected with 2 μg of β-galactosidase control vector. Sixteen hours post-transfection, cells were pretreated for 3 h with NAC (10 mM), exposed to hypoxia or normoxia for 18 h and then harvested. Relative luciferase activity was measured with a luciferase assay system (Promega, Madison, WI) using a luminometer (Turner Design, Sunnyvale, CA). Luciferase activity was normalized for transfection efficiency using β-galactosidase activity, which was measured using a FluoReporter® lac z/β-galactosidase quantitation kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Luciferase activity was assessed as relative light units and used as an indicator of transcriptional induction of NFκB.

**NFκB Activation and Binding.** Specific binding of activated NFκB was measured with a TransAM™ NFκB kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. The oligonucleotide containing the NFκB consensus sequence (5'-GGGACTTTCC-3') is immobilized onto a 96 well plate. Only the active form of NFκB in the cell extract specifically binds to the oligonucleotide. Epitopes on p50, p52, p65, c-Rel and Rel B are accessible only when NFκB is bound to its target DNA. Nuclear extracts (5-10 μg) were added to the wells followed by the primary antibody against p65 and the HRP-conjugated secondary antibody. The optical density was measured at 450-nm with an absorbance plate reader.

**Preparation of BSA-SSG$^{[35}S]$.** BSA-SSG$^{[35}S]$ was prepared as described earlier with the following modifications (22). After the reaction of S-carboxymethyl-BSA with N-succinimidyl
pyridyl bis (3,3'-dithio-propionate) and quenching with glycine, BSA was separated from small molecules either by gel filtration chromatography or by dialysis against two changes of 100 mM sodium phosphate buffer, pH 7.0. The modified BSA was then treated with an excess of \([35S]\) GSH for 1 h at room temperature. The resulting BSA-SSG\([35S]\) product was separated from \([35S]\) GSH as described previously and typically had \(\sim 0.9\) GS-equivalent/mol of BSA (22).

**Radiolabel Assay for Glutaredoxin Activity.** Whole cell lysates were analyzed for Grx activity with a radiolabel assay, which monitors time-dependent release of radioactivity from BSA-SSG\([35S]\) (23). Aliquots of lysate and assay buffer (0.1 M Na\(^+\)/K\(^+\) phosphate buffer, pH 7.4, containing 0.5 mM GSH and 0.2 mM NADPH) were pre-warmed to 30°C and mixed with an aliquot of BSA-SSG\([35S]\) (final concentration of 0.1 mM) to initiate the reaction (total volume 0.5 ml). Aliquots of the reaction mixtures were precipitated with ice-cold trichloroacetic acid (final concentration of 10%) at 12 sec, 1, 2, and 3 min, respectively. After centrifugation, the supernatants were analyzed for \(35S\) by scintillation counting. Total rates of deglutathionylation (slopes of \([35S]\) GSH released versus time) were corrected for non-enzymatic deglutathionylation by subtracting the rate of \([35S]\) GSH released by GSH in the absence of cell lysate. Enzymatic rates were expressed as nmol product/min/mg of protein.

**Nuclear and Cytoplasmic Extracts.** Nuclear and cytoplasmic extracts were prepared according to a modification of the method of Wang et al. (24). Briefly, cells cultured on 150-mm Petri dishes were scraped into 1 ml of ice-cold phosphate buffered saline (PBS). Cells were centrifuged at 1,600 x g for 5 min at 4°C. Cell pellets were lysed in 300 μl of low salt buffer (20 mM HEPES, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl\(_2\), 0.2 mM EDTA, and 0.1% Triton X-100, pH 7.6) on ice. After 20 min incubation, supernatants were collected as cytoplasmic extracts after centrifugation at 4,000 x g for 5 min at 4°C. Nuclear pellets were then resuspended in high salt buffer (20 mM HEPES, 20% glycerol, 500 mM NaCl, 1.5 mM MgCl\(_2\), 0.2 mM EDTA, and 0.1% Triton X-100, pH 7.6) and tubes were rotated for 30 min at 4°C, centrifuged at 16,000 x g and supernatants were saved as nuclear extracts.

**In Vitro Deglutathionylation Assay.** Recombinant Grx1 was isolated as described previously (25). Nuclear extracts were prepared as described above except 10 mM N-ethylmaleimide (NEM) was included in lysis buffer to block all free thiols. NEM-containing nuclear extracts were processed through spin columns (cut off 600 Da) to rid excess free NEM that potentially would inactivate the subsequent enzymatic reaction with Grx. Extracts were divided into different parts. One part was untreated and the other part was subdivided and aliquots were incubated with one of the following: a) 4 mM dithiotheitol (DTT), b) Grx1 plus Grx reducing system/recycling buffer (containing 0.2 mM NADPH, 0.5 mM GSH and 2 units/ml GSSG reductase), c) recycling buffer alone. The assay was done with varying Grx1 concentrations using 1 μM (0.023 units), 100 nM and 10 nM Grx1. The incubation times for DTT and Grx1 were 10 min and 5 min, respectively. Grx1, along with its recycling system, efficiently converts glutathione-protein disulfides to free protein thiols and GSH. NEM binds irreversibly to free sulfhydryl groups but does not react with cysteine residues that are already oxidized. Subsequently, p56-NFκB DNA binding activity was measured by ELISA.

**Western Blot Analysis.** At indicated time points, total cell extracts were prepared in ice-cold RIPA lysis buffer (150 mM NaCl, 1 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100, 50 mM Tris-Cl, pH 7.4) supplemented with a cocktail of protease inhibitors (Roche Diagnostics, Indianapolis, IN). The lysates were centrifuged, and the resulting supernatants were assayed for total protein content (Bio-Rad, Hercules, CA). Equivalent amounts of protein were diluted in sample buffer (200 mM Tris-Cl, 15% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and resolved on sodium dodecyl sulphate-polyacrylamide gel. The proteins were then transferred and immobilized on PVDF membranes (Millipore, Bedford, MA) and probed with appropriate primary and secondary antibodies. Immunodetection was accomplished by an
enhanced chemiluminescence detection system (Pierce, Rockford, IL).

Immunocytochemistry. MIA PaCa-2 cells were cultured on 35-mm glass bottom Petri dishes (MatTek Corporation, Ashland, MA). Cells were fixed with 1% paraformaldehyde for 30 min at room temperature and permeabilized with 0.25% Triton X-100 for 5 min at room temperature. After washes with Tris-buffered saline with 0.1% Tween-20 (TBST), cells were incubated with blocking solution (1% BSA in TBST) for 1 h followed by primary monoclonal antibody against p65 NFκB (1:100) overnight at 4°C. Cells were then washed three times with TBST and incubated with anti-mouse secondary Alexafluor 488 (1:500) for 1 h at room temperature and followed by three washes with PBS. DRAQ-5 (1 µM) was included in the final wash to stain the nuclei. Images of Alexafluor 488 fluorescence were collected using 488-nm excitation and > 505-nm emission filters. DRAQ-5 fluorescence was imaged with a 633-nm excitation light and > 640-nm emission filter using a Zeiss 510 NLO laser scanning confocal microscope.

Caspase-3 Activation. Caspase-3 activity was measured using a Caspase-Glo™ 3/7 kit (Promega) according to the manufacturer’s instructions. At an indicated time point, cultured MIA PaCa-2 cells were collected into a test-tube followed by centrifugation. The pellet was resuspended and lysed with RIPA buffer. Caspase-Glo™ 3/7 reagent and the lysate were mixed in 1:1 ratio, and luminescence was measured with a luminometer. The resulting luminescence is proportional to caspase activity.

Apoptosis Assay. Apoptotic cell death was determined from nuclear morphology after propidium iodide staining in the presence of digitonin. At indicated time points, floating and adherent cells were collected, centrifuged and resuspended in PBS containing 200 µM of digitonin (Calbiochem, La Jolla, CA) and 25 µM of propidium iodide (Molecular Probes, Eugene, OR). Digitonin permeabilizes the plasma membrane and allows propidium iodide to enter cells and stain all nuclei. Thus, propidium iodide staining in the presence of digitonin is equivalent to staining with Hoechst and DAPI, the two commonly used fluorescent dyes in the literature to assess apoptosis on a cell by cell basis. Apoptotic nuclei were scored as apoptotic based on nuclear condensation and fragmentation and counted by a 40X microscope objective using a rhodamine filter set and expressed as a percentage of total cells. At least 200 cells were counted from three different microscopic fields for each sample. In addition, we previously showed that apoptotic death after hypoxia plus NAC treatment assessed by propidium iodide and digitonin correlates closely with apoptotic death assessed with a Cell Death Detection ELISAPLUS kit (Roche Applied Science, Indianapolis, IN), which measures mono- and oligonucleosomes of 180-bp multiples formed in the cytoplasmic fractions during apoptosis (3).

Statistical Analysis. Data are presented as means ± SEM from at least three independent experiments. Differences were assessed by two-tailed paired Student’s t-test with Instat Software (GraphPAD, San Diego, CA). A p value < 0.05 was considered to be statistically significant.

RESULTS

NAC-Induced Apoptosis is Oxygen-Dependent in Pancreatic Cancer Cells. Our previous study showed that NAC, a GSH generating agent, enhanced hypoxic apoptosis in transformed murine embryonic fibroblasts (MEFs) by a mechanism apparently involving GSH-dependent suppression of NFκB transactivation (3). Since several hypoxia-dependent genes are activated within a narrow range of O2 concentration, we wanted to determine the window within which NAC is capable of enhancing hypoxic apoptosis in cancer cells. We exposed MIA PaCa-2 cells to different concentrations of O2 in the presence and absence of NAC (10 mM). Based on direct measurements with an oxygen probe, O2 concentrations in chambers flushed with 95% N2/5% CO2, 0.5% O2/94.5% N2/5% CO2, and 1% O2/94% N2/5% CO2 were 0.6%, 1.6%, and 2.1% O2, respectively. Thus, even in chambers with 95% N2/5% CO2, medium O2 content did not reach zero (anoxia), possibly due to residual O2 in the gas mixture and outgassing of O2 from plastic Petri dishes and other plastic parts inside the chamber. Hypoxia alone for 22 h in 2.1%, 1.6%, and 0.6% O2 induced very little apoptosis (1.9% during normoxia vs. 4.7%, 3.7%, and
3.1%, respectively). In contrast, NAC (10 mM) enhanced hypoxic apoptosis in 
O₂-dependent manner (2.4% during normoxia vs. 10.3%, 19.6%, and 22.6%, respectively) (Fig. 1A).

NAC is rapidly taken up by the cells. Once inside the cells, endogenous esterases release free L-cysteine, which is a precursor of GSH synthesis (26). Like NAC, GSH-ester (5 mM) enhanced apoptosis from 2.4% (normoxia) to 24% after 22 h exposure to hypoxia (1.6% O₂) (Fig. 1B). To confirm that NAC-mediated enhancement of apoptosis in hypoxic cells was due to an increase in GSH, we incubated cells with the D-isomer of NAC (D-NAC), which is not a precursor of GSH synthesis. Unlike the L-isomer of NAC, the D-isomer did not promote hypoxic apoptosis in MIA PaCa-2 cells (Fig. 1B).

NAC activated caspase-3 in hypoxic cells indicative of apoptotic death (Fig. 1C). Poly-ADP ribose polymerase (PARP) is a target of cysteine proteases, and caspase-3 cleaves 116 kDa PARP into a 85 kDa fragment during apoptosis (27). Hypoxia alone did not cause PARP cleavage assessed by Western blot analysis. In contrast, hypoxia in combination with NAC (10 mM) produced the 85 kDa cleavage product of PARP (Fig. 1D). Collectively, the results from Fig. 1 indicate that elevated GSH is responsible for enhancing hypoxic apoptosis in pancreatic cancer cells in agreement with our previous results in MEFs (3).

Inhibition of NFκB activation is Oxygen-Dependent in MIA PaCa-2 cells. Hypoxia alone (1.6% and 0.6% O₂) increased NFκB luciferase activity by 2.4- and 2.8-fold, respectively, compared to normoxia. The increase was blocked by NAC (Fig. 2A). To determine whether blockade of hypoxia-induced transcriptional activation of NFκB was due to decreased p65-NFκB binding to DNA, NFκB DNA binding was measured. Hypoxia (1.6% O₂) alone (18 h) increased p65-NFκB DNA binding 2.1-fold (compared to normoxia), which was abolished by NAC (Fig. 2B). NAC also affected NFκB target genes. In our previous study, we identified XIAP as a target gene for NFκB during hypoxia to MEFs (3). We also confirmed NFκB-dependent activation of XIAP in MIA PaCa-2 cells during hypoxia, which was prevented by NAC (data not shown). The results indicated that NAC treatment inhibited transcriptional activation of NFκB thereby inhibiting the survival of hypoxic cancer cells.

NAC Does Not Change NFκB Protein Levels. NFκB inactivation by NAC may also be due to decreased stability of NFκB protein. To examine this possibility, we determined p65-NFκB protein expression in whole cell lysates after hypoxia with Western blotting. During normoxia, p65-NFκB protein levels were low and not changed by NAC (10 mM) (Fig. 3A). Hypoxia alone (1.6% O₂, 16 h) increased p65-NFκB protein levels, which were not affected further by NAC. Therefore, the capability of NAC to enhance hypoxia-induced apoptotic death did not appear to be due to decreased stability of the NFκB protein (decreased expression or increased degradation) that would then lead to decreased expression of anti-apoptotic proteins. Rather, some other event was regulating NFκB inhibition after NAC treatment of hypoxic MIA PaCa-2 cells.

NAC Does Not Prevent Hypoxia-Induced Nuclear Translocation of NFκB. Previously, we showed that NAC does not prevent hypoxia-induced IκBα degradation in MEFs (3). Here, we further investigated whether NAC affects the nucleo-cytoplasmic shuttling process of NFκB with Western blotting. Hypoxia increased nuclear translocation of p65-NFκB, and NAC did not prevent the translocation (Fig. 3B). To corroborate these results by Western blotting, we performed immunocytochemical analysis of the p65-NFκB after 18 h hypoxia (1.6% O₂). During normoxia, p65-NFκB was primarily localized to the cytoplasm, and large dark voids of NFκB immunofluorescence in confocal images corresponded to DRAQ-5-stained nuclei (Fig. 3C), indicating that NFκB was localized to the cytoplasm and therefore was not activated. Hypoxia induced p65-NFκB translocation to the nucleus, which was not blocked by NAC (Fig. 3C). Thus, NAC did not affect NFκB protein stability or nuclear translocation during hypoxia. Rather, the effect of NAC lays downstream of these events.
Our results so far indicated that NFκB activation was critical for pancreatic cancer cell survival during hypoxia. To confirm independently the importance of NFκB, we determined whether a NFκB inhibitor would enhance hypoxic apoptosis in MIA PaCa-2 cells. Accordingly, we exposed cells to hypoxia (1.6% O₂, 19 h) in the presence and absence of JSH-23 (10 μM). JSH-23 inhibits NFκB DNA binding and transcriptional activation without affecting IκBα degradation (28). Even during normoxia, JSH-23 increased apoptosis from 2% to 5.2%, indicating a survival function of NFκB that is constitutively activated in MIA PaCa-2 cells (Fig. 4). After hypoxia, JSH-23 and NAC increased apoptosis from 3.5% to 15% and 20%, respectively (Fig. 4). JSH-23, however, did not further enhance NAC-induced hypoxic apoptosis. Thus, NAC treatment during hypoxia is sufficient to inhibit NFκB and induce apoptosis. Also, the results indicate that NFκB pathway is crucial in protecting MIA PaCa-2 cells from hypoxia-induced cell death.

**NAC Causes NFκB Inactivation through Sulfhydryl Modification.** p65-NFκB has critical cysteines in its DNA-binding domain. To determine whether p65-NFκB undergoes sulfhydryl modification, MIA PaCa-2 cells were exposed to hypoxia (1.6% O₂) for 18 h with and without 10 mM NAC. Subsequently, cells were treated with NEM (10 mM) to adduct free thiols and prevent alterations in thiol-disulfides during cell lysis and preparation of nuclear extracts. The extracts were then processed through spin columns (600 Da cut off) to rid excess NEM that would inactivate the later enzymatic reactions with GRx. Subsequently, extracts were divided into four parts. One part was untreated, and the other three parts were incubated with a) DTT (4 mM), b) GRx1 plus recycling buffer (GSH [0.5 mM], NADPH [0.2 mM] and GSSG reductase [2 U/ml]), and c) recycling buffer alone. NFκB DNA binding activity was then measured after each of the treatments.

NEM binds irreversibly to free sulfhydryl groups but does not react with oxidized sulfhydryl. DTT (a disulfide reducing agent) does not restore NEM-adducted thiols but does convert disulfides to free thiols. After hypoxia, DTT (4 mM) did not increase NFκB DNA binding after NEM treatment, indicating that most sulfhydryls in the NFκB protein were in the reduced and activated form after hypoxia (Fig. 5A). After hypoxia plus NAC, however, DTT increased NFκB DNA binding by 3.2-fold compared to hypoxia alone (Fig. 5A). This result indicated that critical sulfhydryls in NFκB required for DNA binding were converted to disulfides after hypoxia plus NAC, since reduction of these disulfides by DTT restored NFκB DNA binding.

To determine whether S-glutathionylation contributes to NFκB inactivation by NAC, NEM-treated cell lysates were incubated with the GRx system (GRx1, GSH, GSSG reductase and NADPH) for 10 min before measurement of NFκB DNA binding capacity. GRx1 is a specific catalyst of protein-SSG reduction coupled to the GSH recycling system which maintains GRx and GSH in their reduced forms. Thus, effects of GRx in this system are diagnostic of functional modifications of proteins via S-glutathionylation (protein-SSG) (29-31). The complete GRx system increased NFκB DNA binding in hypoxia plus NAC-treated samples as compared to hypoxia (Fig. 5B). However, the GSH recycling system in the absence of GRx1 was without effect (Fig. 5C). As little as 10 nM GRx1 reactivated the NFκB (Fig. 5D). Since GRx specifically catalyzes reduction of protein-SSG mixed disulfides, these results support the conclusion that NFκB becomes S-glutathionylated during hypoxia plus NAC treatment to produce NFκB inactivation. Moreover, ratios of NFκB DNA binding for hypoxia plus NAC to hypoxia alone in DTT-treated and GRx-treated samples were indistinguishable (Fig. 5E). This indicates that GRx reverses the inhibition of NFκB DNA binding to the same extent as DTT, which supports the conclusion that NAC-mediated thiol modification of NFκB is mostly due to S-glutathionylation.

**GRx activity is required for p65-NFκB-SSG formation.** Several proteins have been shown to undergo GRx-facilitated S-glutathionylation in the presence of GS" (20). First we determined whether hypoxia and/or NAC modulates cellular GRx activity. MIA PaCa-2 cells were incubated for 18 h under
normoxic or hypoxic conditions in the presence and absence of NAC. None of the treatments, including hypoxia plus NAC, caused an irreversible change in the endogenous activity of GRx (Fig. 6).

To determine whether p65-NFκB glutathionylation is affected by steady state GRx activity, we generated a stable GRx knock-down cell line by infecting Mia PaCa-2 cells with GRx1-shRNA or ns-shRNA. Cells infected with GRx1-shRNA showed a substantial decrease (60%) in GRx1 protein levels (Fig. 7A) and a corresponding decrease (77%) in GRx1 activity (Fig. 7B) compared to cells containing ns-shRNA. In ns-shRNA cells, NAC decreased hypoxia-induced p65-NFκB DNA binding (Fig. 7C) and increased hypoxic apoptosis (Fig. 7D). By contrast, in GRx1-shRNA cells, NAC treatment did not decrease hypoxia-induced p65-NFκB DNA binding (Fig. 7C), and increased hypoxic apoptosis only by 3% (Fig. 7D). Thus, knock-down of GRx1 protein was sufficient to prevent NAC-induced NFκB inactivation and apoptosis during hypoxia consistent with a direct link between NFκB S-glutathionylation and hypoxic apoptosis (see Discussion).

DISCUSSION

The main conclusion of the present study is that GSH supplementation promotes hypoxic apoptosis in pancreatic cancer cells by suppressing the NFκB survival pathway. The checkpoint for NFκB inhibition does not lie at the level of IκBα degradation or NFκB nuclear translocation, as suggested by some studies (32;33). Instead, NAC enhances hypoxic apoptosis by inhibiting the binding of p65-NFκB to DNA. We interpret this event to be largely due to GRx-dependent S-glutathionylation of p65-NFκB. This is the first evidence that GSH supplementation can promote S-glutathionylation of p65-NFκB and apoptosis in hypoxic cancer cells.

A previous report shows that depending upon the nature of the stimulus, NAC either promotes or suppresses apoptosis independently of changes in GSH levels and redox status (34). However, we have previously shown that NAC-induced hypoxic apoptosis was not a consequence of an antioxidant effect but rather due to an increase of GSH content per se in MEFs (3). Here we showed that NAC and GSH-ester, both agents increasing intracellular GSH, promoted hypoxic apoptosis in pancreatic cancer cells (Fig. 1A). In contrast, D-NAC, which increases total cellular D-cysteine content but does not support GSH biosynthesis, did not enhance hypoxic apoptosis (Fig. 1B), confirming our previous findings in MEFs that NAC-mediated enhancement of apoptosis occurs via elevated levels of intracellular GSH (3).

GSH is the major thiol-disulfide redox buffer of the cell (35). Under pro-oxidant conditions, GSH itself can bind covalently to reactive cysteine thiols of various proteins to form mixed disulfides, a process termed S-glutathionylation (17;36). Many transcription factors, including NFκB, have sensitive thiols that respond to changes in redox environment. Reduction of these sulfhydryls enhances transcriptional activation whereas oxidation inhibits activation. Hypoxia caused p65-NFκB activation and DNA binding, which was blocked by NAC (Fig. 2). Since D-NAC and non-thiol antioxidants like trolox did not block p65-NFκB DNA binding (data not shown), the results indicated that the NAC-mediated effect on p65-NFκB was GSH-dependent. These results were also in agreement with recent reports showing that NAC and increased GSH down-regulate cigarette smoke- and tumor necrosis factor-induced NFκB activation in A549 alveolar epithelial, U937 human histiocytic lymphoma, HeLa human epithelial, and MCF-7 human breast cancer cells (37;38). Our results also indicate that NFκB pathway is crucial in protecting MIA PaCa-2 cells from hypoxia-induced cell death, as confirmed by pro-apoptotic effect of the NFκB inhibitor (Fig. 4).

To explore the nature of the sulfhydryl modification of the p65-NFκB protein, we determined DNA binding of p65-NFκB in vitro. After treatment with NEM, p65-NFκB DNA binding in nuclear extracts of hypoxic cells was not increased by further treatment with DTT, suggesting that most sulfhydryls affecting p65-NFκB DNA binding were in a reduced (NFκB-SH) form after hypoxia and therefore susceptible to alkylation by NEM. In contrast after hypoxia plus NAC followed by NEM, DTT treatment
increased p65-NFκB DNA binding capacity (Fig. 5A) suggesting that NAC led to modification of the sulfhydryls of the p65-NFκB that are responsible for DNA binding. DTT is a general reducing agent and does not provide information regarding the nature of the sulfhydryl modification. However, GRx1, which specifically de-glutathionylates protein-SSG mixed disulfides (17;23), also restored p65-NFκB DNA binding capacity in nuclear extracts of cells treated with hypoxia plus NAC (Fig. 5B,D). Moreover, the reactivation ratio for the GRx system matched that for DTT (Fig. 5E), which suggested that most of the p65-NFκB sulfhydryl modification was p65-NFκB-SSG. Although NAC also increases intracellular L-cysteine concentrations, the possibility that formation of p65-NFκB-SSCysteine is responsible for NFκB inactivation can be ruled out since GRx does not catalyze removal of cysteine from the protein-SSCysteine (14;17;23). Furthermore GSH-ester, which does not increase cellular L-cysteine levels, also enhanced hypoxic apoptosis (Fig. 1B), speaking against the involvement of protein-SSCysteine.

In addition to protein-SSG formation, protein thiols can form intermolecular disulfide linkages between different proteins during oxidative stress (39). Such modifications are reduced by DTT, but they are not sensitive to GRx-catalysis. Furthermore in Western blots of p65, we did not detect higher molecular weight bands under non-reducing conditions (data not shown). Thus, formation of intermolecular disulfides on p65 is unlikely to be responsible for NAC-mediated NFκB inactivation during hypoxia.

Unless intracellular GSSG concentrations reach unusually high levels (i.e., high levels of oxidant stress where GSH/GSSG = 1), GSSG is unlikely to react to form protein-SSG based on typical redox potentials of cysteine residues in proteins (40). Consequently, GS-NO and GS• have been proposed as alternative GS-donors for protein-SSG formation under conditions where GSSG does not accumulate (11;20;41;42). Hypoxia is known to induce formation of reactive oxygen species, such as superoxide (O2•⁻) and H2O2, that can be converted to hydroxyl radicals (OH•) through the iron-catalyzed Fenton reaction (43). OH• is also generated without iron through peroxynitrite from the interaction between nitric oxide (NO) and O2•⁻, both of which may be produced in hypoxia (43). GSH scavenges OH• to form the glutathione thyl radical GS• (20). GS• can also be formed from GS-NO (44). In the presence of a GS• generating system, GRx accelerates S-glutathionylation of proteins, apparently by forming a stabilized GRx-S-S-glutathione disulfide anion radical intermediate (GRx-SSG•⁻) (20). The GRx-SSG•⁻ intermediate is converted to the reduced enzyme (GRx-SH) mainly by sequential reaction with O2 and then GSH (20). Thus, hypoxia would favor GRx-SSG•⁻ accumulation and subsequent transfer of the glutathionyl moiety from GRx-SSG•⁻ to form protein-SSG, as shown previously for GAPDH, actin, and PTP1B (20). This hypothesis would be consistent with our previous findings that NAC increases intracellular GSH by ~45% during hypoxia (3) creating conditions conducive to GS• generation, and that NAC-induced NFκB transcriotional inactivation and apoptosis were O2•⁻-dependent (Fig. 1A and 2A).

Further support for the involvement of GRx in NAC-induced NFκB inhibition and apoptosis is provided by the GRx1 knock-down experiment. Although GRx1 activity by itself was not affected by the NAC treatment (Fig. 6), GRx1 protein knock-down prevented NAC-induced inhibition of p65-NFκB DNA binding (Fig. 7C) and hypoxic apoptosis (Fig. 7D). Taken together, these data support the conclusion that GRx-mediated p65-NFκB S-glutathionylation is a crucial event in GSH-mediated hypoxic apoptosis in cancer cells.

GRx1 lacks known nuclear translocation motifs and is localized primarily to the cytosol (45). This finding suggests that GRx1-dependent p65-NFκB-SSG formation occurs in the cytosol and that the p65-NFκB-SSG is further transported to the nucleus where it is unable to bind to DNA. However, the exact site of the p65-NFκB-SSG formation remains to be determined. In a recent study, oxidative stress with H2O2 inhibited NFκB nuclear translocation and activation in lung cells, and this was attributed to inactivation of IκB kinase (IKK)
via S-glutathionylation of Cys 179 of IKK, most likely resulting from initial formation of IKK-sulfenic acid (46). However, NAC treatment during hypoxia in the present study did not prevent IκBα degradation (3) or p65-NFκB nuclear translocation (Fig. 3), which is inconsistent with IKK inhibition. These findings indicate that IKK-β glutathionylation most likely did not play an important role in NAC-mediated NFκB inhibition during hypoxia in the pancreatic cancer cells.

In summary, we identified GRx-dependent S-glutathionylation of p65-NFκB as a major mechanism underlying the inhibition of the NFκB survival pathway and promotion of apoptosis after GSH supplementation in hypoxic pancreatic cancer cells. Accordingly, we propose the following model (Fig. 8). Hypoxia activates NFκB providing a survival signal against hypoxia-induced cell death. With enhanced intracellular GSH, OH* generated during hypoxia is scavenged by GSH (NAC) to form GS*. GRx1 reacts with GS* to form a GRx-SSG* intermediate (20), and this intermediate transfers GS* to form p65-NFκB-SSG, a reaction that would be enhanced under hypoxic conditions (20). S-glutathionylation then inactivates p65-NFκB leading to cell death. Although the contribution of GS* to p65-NFκB-SSG formation requires further investigation, our model would explain the observations that GRx1 knock-down prevents NAC-induced NFκB inhibition and hypoxic apoptosis. Finally, the data suggesting that GSH supplementation enhances only hypoxic apoptosis have therapeutic implications, and administration of NAC may be a strategy to circumvent resistance to chemotherapy and radiation therapy in solid tumors whose core is often hypoxic. NAC treatment is attractive since NAC is already shown to be safe and effective for treatment of patients with acetaminophen overdose.
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**FOOTNOTES**

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1Abbreviations used: BSA, bovine serum albumin; D-NAC, N-acetyl-D-cysteine; DTT, dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione (reduced form); GSSG, glutathione disulfide (oxidized form); GRx, glutaredoxin; GRx-SSG•-, GRx-S-S-glutathione disulfide anion radical, GS•, glutathionyl thyl radical; HEPES, N-(2-hydroxyethyl) piperazine-N’-2-ethane sulfonic acid; HRP, horseradish peroxidase; IKK, IkB kinase; IkB, inhibitor of nuclear factor κB; MEFs, murine embryonic fibroblasts; NAC, N-acetyl-L-cysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NEM, N-ethylmaleimide; NFκB, nuclear factor κB; NO, nitric oxide; O2•-, superoxide; OH•, hydroxyl radical; PARP, poly-ADP ribose polymerase; PBS, phosphate-buffered saline; PTP1B, protein tyrosine phosphatase 1B; RHR, Rel homology region; TAD, transcription activation domain; TBST, Tris-buffered saline with Tween-20; XIAP, X chromosome-linked inhibitor of apoptosis protein.

**FIGURE LEGENDS**

Fig. 1. **NAC-induced apoptosis is oxygen- and GSH-dependent.** *A*, MIA PaCa-2 cells were pre-incubated in the presence and absence of 10 mM NAC for 3 h followed by exposure to 0.6-21% O2 for 22 h. Subsequently, cells were harvested and scored for apoptosis with propidium iodide and digitonin as described in Experimental Procedures. ‡, p < 0.001 compared to hypoxia alone. *B*, cells were pre-
incubated with 10 mM NAC, 10 mM D-NAC, 5 mM GSH-ester or no addition for 3 h followed by 22 h exposure to hypoxia (1.6% O₂). Cells were then scored for apoptosis as in A. ‡, p < 0.001 compared to hypoxia alone. C, cells were treated with 10 mM NAC or no addition followed by hypoxia (0.6% O₂) for 18 h. Samples were prepared for caspase-3 measurements, as described in Experimental Procedures. z-VAD-fmk (50 μM) was included as a negative control. Results are expressed as fold increase over normoxia (N). *, p < 0.05 compared to hypoxia (H).

Fig. 2. Inhibition of NFκB transactivation is oxygen-dependent. A, MIA PaCa-2 cells were cotransfected with NFκB luciferase reporter construct or empty vector (EV) and β-galactosidase for 16 h. Cells were then exposed to different concentrations of O₂ (21%, 1.6% and 0.6%) in the presence and absence of 10 mM NAC for 18 h. Cells were exposed to tumor necrosis factor α (TNF) for 6 h as a positive control. Luciferase activity was normalized against β-galactosidase activity. Normoxic (21% O₂) value was taken as 1. #, p < 0.01 compared to normoxia. B, cells were incubated in the presence and absence of 10 mM NAC for 3 h followed by normoxia (N, 21% O₂) or hypoxia (H, 1.6% O₂) for 18 h. Binding of p65-NFκB to its consensus DNA sequence in nuclear extracts was assessed by ELISA as described in Experimental Procedures.

Fig. 3. NAC does not affect protein level or hypoxia-induced nuclear translocation of NFκB. A, MIA PaCa-2 cells were treated with 10 mM NAC or no addition for 3 h followed by normoxia (N) or hypoxia (H, 1.6% O₂) for 16 h. Total cell extracts were probed for p65-NFκB by Western blotting. B, cells were exposed to normoxia (N) or hypoxia (H, 1.6% O₂) for 18 h in the presence and absence of 10 mM NAC. Cytoplasmic and nuclear fractions were prepared as described in Experimental Procedures, and p65-NFκB proteins were analyzed with Western blotting (15 and 30 μg of protein/lane for cytoplasmic and nuclear extracts, respectively). YY-1 and actin were used as a nuclear marker and loading control, respectively. C, cells were pretreated with 10 mM NAC for 3 h followed by hypoxia (1.6% O₂) for 18 h. Samples were prepared for immunocytochemistry using anti-p65 antibody and Alexa 488-conjugated anti-mouse IgG (green). DRAQ-5 dye (red) was included to stain the nuclei. Shown are representative confocal images. Scale bar is 10 μm.

Fig. 4. NAC-induced hypoxic apoptosis is not enhanced by NFκB inhibitor. MIA PaCa-2 cells were pre-incubated with JSH-23 (1 h, 10 μM) and NAC (3 h, 10 mM) alone or together with JSH-23 and NAC. Cells were then exposed to hypoxia (1.6% O₂) or normoxia for 19 h. Subsequently, cells were harvested and scored for apoptosis with propidium iodide and digitonin as described in Experimental Procedures.

Fig. 5. NAC causes NFκB inactivation through sulfhydryl modification. MIA PaCa-2 cells were exposed to normoxia (N) and hypoxia (H, 1.6% O₂) in the presence and absence of 10 mM NAC for 18 h. Cultures were treated with 10 mM NEM to prevent any thiol/disulfide changes during processing, nuclear extracts were prepared and spun through the spin columns (600 Da cut off) to rid excess free NEM. Extracts were divided into different parts as described in the Experimental Procedures. Samples were then incubated with none, 4 mM DTT (A), 1 μM GRx plus recycling buffer (0.2 mM NADPH, 0.5 mM GSH and 2 units/ml GSSG reductase) (B), or recycling buffer alone (C). p65-NFκB DNA binding activity was measured with ELISA. Absorbance value of normoxia without DTT (A), GRx/recycling buffer (B) or recycling buffer alone (C) treatment was set to 1. *, p < 0.05 compared to H; **, p < 0.01 compared to H. D, cells were exposed to hypoxia (H, 1.6% O₂) in the presence and absence of 10 mM NAC for 18 h. NEM treated nuclear extracts were incubated with the GRx system (GRx, GSH, GSSG reductase and NADPH) for 10 min. Concentrations of GRx (0 nM, 10 nM, 100 nM and 1 μM) were used for the assay. p65-NFκB DNA binding activity was measured with ELISA. Absorbance value without
treatment was set to 1. E, The values for hypoxia plus NAC (H+NAC) were divided by the values for hypoxia alone (H) after DTT and GRx/Recycling treatment and expressed as ratios of H+NAC/H.

**Fig. 6. NAC does not change glutaredoxin activity.** MIA PaCa-2 cells were pre-incubated for 3 h in the presence and absence of NAC (10 mM). Cells were then exposed to hypoxia (H, 1.6% O$_2$) or normoxia (N) for 18 h. Subsequently, cells were lysed and GRx activity was measured in whole cell lysates with a radiolabel assay, which monitors time-dependent release of radioactivity from BSA-SSG[35S] as described in *Experimental Procedures*. GRx activity is expressed as nmol of [35S]GSH released/min/mg of protein.

**Fig. 7. GRx1 is required for p65-NFκB S-glutathionylation.** A, Immunoblots of steady state GRx1 and actin protein levels from whole cell extracts of MIA PaCa-2 ns-shRNA and GRx1-shRNA cell lines. Relative GRx1 protein levels were calculated by densitometric analysis using actin as a loading control. Protein levels from ns-shRNA cells were set to 1.0. B, GRx1 enzymatic activities were measured and calculated for ns-shRNA and GRx1-shRNA cells, as described in Fig. 6. Enzymatic activities in ns-shRNA cells were set to 100%. C, MIA PaCa-2 ns-shRNA and GRx1-shRNA cells were exposed to hypoxia (H, 1.6% O$_2$) for 18 h in the presence and absence of NAC (10 mM). Nuclear extracts were prepared and p65-NFκB DNA binding was measured with ELISA. Results are expressed as fold increase over normoxic ns-shRNA cells. *, $p < 0.05$ compared to ns-shRNA+NAC in hypoxia. D, apoptosis was assessed after 22 h exposure to hypoxia (H, 1.6% O$_2$) as described in Fig. 1. *, $p < 0.0001$ compared to ns-shRNA+NAC in hypoxia.

**Fig. 8. Proposed model of inhibition of the NFκB survival pathway by S-glutathionylation of p65-NFκB.** See text for details.
Figure 1

A. Bar graph showing the effect of NAC on apoptosis under different O₂ concentrations.

B. Bar graph showing the effect of GSH-ester on apoptosis under normoxia and hypoxia conditions.

C. Graph showing the fold increase in Caspase-3 activity under different conditions.

D. Western blot analysis showing the expression levels of 116 kDa, 85 kDa, and Actin proteins under normoxia and hypoxia conditions with or without NAC and Z-VAD treatment.
Figure 2

A

Luciferase (Fold Increase)

- NAC  +NAC

O₂ Concentration (%)

21  1.6  0.6  TNF  EV

B

NFκB DNA Binding (Fold Increase)

- NAC  +NAC

N  H

*
Figure 3
Figure 4
Figure 5
Figure 6

Graph showing Glutaredoxin Activity (nmol/min/mg protein) for different conditions: N, N+NAC, H, H+NAC.
Figure 7
Hypoxia

\[ \text{OH}^• \]

GSH (NAC)

\[ \text{GS}^• \]

GRx1

GRx1-shRNA

GRx-SSG

Activated NFkB (p65-Cys-SH)

Survival

Inactive NFkB (p65-Cys-SSG)

Cell Death

GRx system

or DTT

Figure 8
Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NFκB
Suparna Qanungo, David W. Starke, Harish V. Pai, John J. Mieyal and Anna-Liisa Nieminen

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