N-Acetyl-l-cysteine Enhances Apoptosis through Inhibition of Nuclear Factor-κB in Hypoxic Murine Embryonic Fibroblasts*

Suparna Qanungo, Mi Wang, and Anna-Lisa Nieminen‡
From the Department of Anatomy and Case Comprehensive Cancer Center, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

In this study, we investigated the role of reduced glutathione (GSH) and nuclear factor-κB (NFκB) in hypoxia-induced apoptosis. Hypoxia caused p53-dependent apoptosis in murine embryonic fibroblasts transfected with Ras and E1A. N-Acetyl-l-cysteine (NAC) but not other antioxidants, such as the vitamin E analog trolox and epigallocatechin-3-gallate, enhanced hypoxia-induced caspase-3 activation and apoptosis. NAC also enhanced hypoxia-induced apoptosis in two human cancer cell lines, MIA PaCa-2 pancreatic cancer cells and A549 lung carcinoma cells. In murine embryonic fibroblasts, all three antioxidants blocked hypoxia-induced reactive oxygen species formation. NAC did not enhance hypoxia-induced cytochrome c release but did enhance poly-(ADP ribose) polymerase cleavage, indicating that NAC acted at a post-mitochondrial level. NAC-mediated enhancement of apoptosis was mimicked by incubating cells with GSH monoester, which increased intracellular GSH similarly to NAC. Hypoxia promoted degradation of an inhibitor of κB (IκB), NFκB-p65 translation into the nucleus, NFκB binding to DNA, and subsequent transactivation of NFκB, which increased X chromosome-linked inhibitor of apoptosis protein levels. NAC failed to block degradation by IκBα and sequestration of the p65 subunit of NFκB to the nucleus. However, NAC did abrogate hypoxia-induced NFκB binding to DNA, NFκB-dependent gene expression, and induction of X chromosome-linked inhibitor of apoptosis protein. In conclusion, NAC enhanced hypoxic apoptosis by a mechanism apparently involving GSH-dependent suppression of NFκB transactivation.

Hypoxia is a frequent feature of rapidly growing malignant tumors and their metastases. Tissue hypoxia resulting from inadequate blood supply generally occurs at early stages of tumor development, beginning at a tumor diameter of only a few millimeters (1). In solid tumors, hypoxia causes a selection of mutations that makes cells resistant to apoptosis and less responsive to cancer therapy (2, 3). Hypoxia also induces genes that protect cells against apoptosis. One of these genes is nuclear factor-κB (NFκB).1 NFκB is a critical transcription factor that tips the balance from apoptosis of cells to proliferation and malignant growth of tumor cells. NFκB belongs to the Re1 family, which includes five mammalian Re1/NFκB proteins: Re1A (p65), c-Re1, Re1B, NFκB1 (p50/p105), and NFκB2 (p52/p100) (4). The activity of NFκB is regulated by movement between the cytoplasm and nucleus in response to cell stimulation (5). NFκB dimers containing Re1A or c-Re1 are retained in the cytoplasm through interaction with inhibitor of κB (IκB) repressor proteins (IκBα, IκBβ, IκBγ, and IκBε) (6). Potent NFκB activators, such as tumor necrosis factor-α, interleukin-1, and lipopolysaccharide, cause IκB kinase (IKK)-mediated phosphorylation of IκBα on serine 32 and serine 36 residues, followed by ubiquitination at the nearby lysine residues and proteasomal degradation (7). This modification of IκBα exposes the nuclear localization signal of NFκB and leads to NFκB translocation to the nucleus, presumably as a consequence of binding to karyopherins (8), proteins responsible for nucleocytoplasmic transport in cells.

An alternative, less-characterized pathway of NFκB activation is by tyrosine 42 phosphorylation of IκBα after hypoxia/reoxygenation or pervanadate treatment (6). However, in T-cells and bone marrow macrophages, tyrosine phosphorylation of IκBα is insufficient to activate NFκB, suggesting that tyrosine and serine kinases act at multiple levels to dissociate the IκBα/NFκB complex (8). Once IκBα is inactivated, NFκB dimers are free to localize to the nucleus, where they undergo further modification, mostly through phosphorylation of the Re1 proteins (9). Activated NFκB in the nucleus binds to promoters of its target genes to initiate the transcription of several genes involved in immune responses, inflammation, viral infection, and cell survival (7, 10). NFκB suppresses apoptosis by inducing expression of various anti-apoptotic genes, including Bcl-xL, Bcl-2, hematopoietic-specific Bcl-2 homolog A1 (Bfl1) (11), caspase-8-FADD-like-interleukin-1-β-converting enzyme inhibitory protein (c-FLIP), tumor necrosis factor receptor-associated factor 1 (TRAF1), tumor necrosis factor receptor-associated factor 2 (TRAF2), and cellular inhibitors of apoptosis (c-IAPs) (7). NFκB also regulates X chromosome-linked IAP (XIAP/hIAP), which inhibits caspase-3 and caspase-7 and prevents activation of pro-caspase-9 (12).

Hypoxia promotes reactive oxygen species (ROS) formation in a number of cell types (13–15). ROS, such as H2O2, may act as second messengers activating NFκB (16). Hyperoxia, which also induces ROS generation, causes nuclear translocation and activation of NFκB.

Received for publication, June 16, 2004, and in revised form, August 19, 2004
Published, JBC Papers in Press, September 16, 2004, DOI 10.1074/jbc.M406749200

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
N-Acetyl-L-cysteine Potentiates Hypoxia-induced Apoptosis

To determine N-acetylcycteine (NAC) effects, cells were transfected with 1 µg of plasmid containing 2 µg of empty vector luciferase control vector. After 16 h, cells were exposed to hypoxia or normoxia for 4 h and then harvested. Relative luciferase activity was measured with a luciferase assay system (PromeGa, Madison, WI) using a luminometer (Turner Design). Luciferase activity was normalized for transfection efficiency using β-galactosidase activity, which was measured using a Fluorometric β-galactosidase quantitation kit (Molecular Probes) according to the manufacturer’s instructions. Luciferase activity was assessed as relative light units and used as an indicator of transcriptional induction of NAC

Caspase-3 Activation—Caspase-3 activity was measured using a commercial kit (R&D Systems, Minneapolis, MN). Briefly, at the indicated time points, cultured MEFs were collected into a test tube, followed by centrifugation. The pellet was resuspended in a lysis buffer provided by the kit. Cell lysates were incubated with a caspase-3 fluorogenic substrate, DEVD-AFC (50 µM). Caspase-3 cleaves DEVD-AFC into free AFC, the fluorescence of which was measured with a CytOxter 2000 fluorescence plate reader using 405-nm excitation and 505-nm emission light. Caspase-3 enzymatic activity was expressed as pmol/mg/min AFC formed.

ROS Formation—Cultured cells were loaded with 10 µM 2',7'-dichloro-7-nilodihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes) for 1 h before exposure to hypoxia. Inside cells, esterases cleave the acetate ester to release free CM-H2DCF, which is non-fluorescent. After oxidation, CM-H2DCF is converted to green-fluorescing dichloro-7-nilodihydrofluorescein (DCF) (25). After hypoxia, medium containing CM-HDCFDA was replaced, and cells were fixed and permeabilized with phosphate-buffered saline (PBS) containing 50% ethanol and 0.1% Tween 20. Subsequently, the cell extracts were centrifuged, and supernatants were collected. DCF fluorescence was measured with a fluorescence plate reader using 450-nm excitation and 530-nm emission filters. The absolute value of DCF—CM-H2DCF-DA was measured by high-performance liquid chromatography (Hewlett-Packard series 1050). MEFs were washed with PBS and lysed using two freeze-thaw cycles. Cells were then scraped, suspended in buffer containing 0.1 mM sodium phosphate, 5 mM EDTA, pH 8.0, and centrifuged. Supernatants were mixed with metaphosphoric acid to a final concentration of 1% to precipitate protein. After centrifugation at 12,000 × g for 15 min, supernatants were diluted in a mobile containing 10 mM sodium phosphate and 69 mM monochloroacetic acid (pH adjusted to 2.72 with NaOH). GSH was resolved on a C18 reverse phase column (Yvad) and detected using electrochemical detection. A standard curve was generated using pure GSH, and results were calculated as nmol GSH/mg of protein (26).

Western Blot Analysis—At the indicated time points, total cell extracts were prepared in ice-cold RIPA lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 50 mM Tris-Cl, pH 8.0) supplemented with a mixture 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% beta-mercaptoethanol, pH 6.8) and resolved on SDS-PAGE gel.

MATERIALS AND METHODS

Cells and Reagents—Monoclonal anti-NFκB p65, polyclonal anti-IκB, anti-actin, anti-c-FLIP, and secondary horseradish peroxidase-linked antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Bcl-xL antibody was from Cell Signaling Technologies (Beverly, MA). Monoclonal antibodies against poly(ADP ribose) polymerase (PARP), cytochrome c, F16-APase, XIAP, and Bcl-2 were purchased from Trevigen (Gaithersburg, MD), PharMingen (San Diego, CA), Molecular Probes (Eugene, OR), and BD Transduction Labs (San Jose, CA), respectively. Hoechst 33342, and the anti-mouse IgG were from Molecular Probes. α-Methylmercurialide was obtained from Calbiochem (La Jolla, CA). The luciferase reporter construct pNFKB-B-Luc was a gift from Dr. Santanu Bose (Cleveland Clinic Foundation, Cleveland, OH), and N-acetylcycteine (NAC) was a gift from Dr. Ian Cotgreave (AstraZeneca, Stockholm, Sweden). All other chemicals were purchased from Sigma.

Chemicals and Reagents—Cell Culture—MEFs, MIA PaCa-2, and A549 human lung carcinoma cells were grown in complete culture medium supplemented with 10 mM glucose. Petri dishes coated with 0.1% gelatin (Sigma). MIA PaCa-2 and A549 cells were grown to 60–70% confluence. Petri dishes were incubated with 0.2% because of osmotic considerations. MEFs were cultured on Primaria medium (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was included in the final medium (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was included in the final medium. At the indicated time points, cultured MEFs were collected into a test tube, followed by centrifugation. The pellet was resuspended in a lysis buffer provided by the kit. Cell lysates were incubated with a caspase-3 fluorogenic substrate, DEVD-AFC (50 µM). Caspase-3 cleaves DEVD-AFC into free AFC, the fluorescence of which was measured with a CytOxter 2000 fluorescence plate reader using 405-nm excitation and 505-nm emission light. Caspase-3 enzymatic activity was expressed as pmol/mg/min AFC formed.

ROS Formation—Cultured cells were loaded with 10 µM 2',7'-dichloro-7-nilodihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes) for 1 h before exposure to hypoxia. Inside cells, esterases cleave the acetate ester to release free CM-H2DCF, which is non-fluorescent. After oxidation, CM-H2DCF is converted to green-fluorescing dichloro-7-nilodihydrofluorescein (DCF) (25). After hypoxia, medium containing CM-HDCFDA was replaced, and cells were fixed and permeabilized with phosphate-buffered saline (PBS) containing 50% ethanol and 0.1% Tween 20. Subsequently, the cell extracts were centrifuged, and supernatants were collected. DCF fluorescence was measured with a fluorescence plate reader using 450-nm excitation and 530-nm emission filters. The absolute value of DCF—CM-H2DCF-DA was measured by high-performance liquid chromatography (Hewlett-Packard series 1050). MEFs were washed with PBS and lysed using two freeze-thaw cycles. Cells were then scraped, suspended in buffer containing 0.1 mM sodium phosphate, 5 mM EDTA, pH 8.0, and centrifuged. Supernatants were mixed with metaphosphoric acid to a final concentration of 1% to precipitate protein. After centrifugation at 12,000 × g for 15 min, supernatants were diluted in a mobile containing 10 mM sodium phosphate and 69 mM monochloroacetic acid (pH adjusted to 2.72 with NaOH). GSH was resolved on a C18 reverse phase column (Yvad) and detected using electrochemical detection. A standard curve was generated using pure GSH, and results were calculated as nmol GSH/mg of protein (26).

Western Blot Analysis—At the indicated time points, total cell extracts were prepared in ice-cold RIPA lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 50 mM Tris-Cl, pH 8.0) supplemented with a mixture 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% beta-mercaptoethanol, pH 6.8) and resolved on SDS-PAGE gel.

The proteins were then transferred and immobilized onto polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with appropriate primary and secondary antibodies. Immunodetection was accomplished by an enhanced chemiluminescence detection system (Fierce).

Immunocytochemistry—At the indicated time points, MEFs cultured on gelatin-coated plastic coverslips were fixed with 3.7% formaldehyde for 30 min at room temperature. Subsequently, cells were rinsed with PBS and permeabilized with 0.25% Triton X-100 for 5 min. After two washes with PBS, cells were incubated with blocking solution (1% bovine serum albumin and 0.1% Tween 20 in PBS) for 1 h, followed by primary monoclonal antibody against p65 NFκB (1:100) overnight at 4 °C. Cells were then washed three times with PBS and incubated with anti-mouse secondary Alexafluor 488 (1:100) and then washed three more times with PBS. Hoechst 33342 (0.5 µg/ml) was included in the final wash to stain the nuclei. Coverslips were attached on slides, mounting medium (Kirkgeagaar & Perry Laboratories, Gaithersburg, MD) was added, and the preparation was covered with a glass coverslip. Images of Alexafluor 488 fluorescence were collected using 488-nm excitation and 505-nm emission filters with a 720-nm emission light filter using a Zeiss 510 NLO laser scanning confocal microscope.

Cytotoxic, Mitochondrial, and Nuclear Extracts—Nuclear and cytoplasmic extracts were prepared according to a modified method by Wang et al. (27). Briefly, cells cultured on 150-mm Petri dishes were scraped into ice-cold phosphate-buffered saline, centrifuged, and washed with five
packed cell volumes of buffer A (250 mM sucrose, 20 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid, 10 mM KC1, 1.5 mM MgCl2, and 1 mM EDTA, pH 7.5). Cells were then resuspended in three to four packed cell volumes of buffer A and homogenized in a glass Dounce homogenizer. Nuclei were pelleted by centrifugation at 10,000 × g for 10 min at 4 °C. Supernatants were mixed with 0.5 volume of buffer B (75 mM Tris-Cl, 20% v/v glycerol, 300 mM KC1, and 600 mM EDTA, pH 7.5) and clarified by centrifugation at 100,000 × g for 30 min at 4 °C to obtain the soluble cytoplasmic fraction (S-100). Nuclear pellets were then resuspended in buffer C (420 mM KCl, 20 mM Tris-Cl, 20% v/v glycerol, and 1.5 mM MgCl2, pH 7.5), tubes were nuted for 30 min at 4 °C and centrifuged at 16,000 × g, and the supernatants were saved as nuclear extracts. Isolation of highly enriched mitochondrial and cytosolic fractions was carried out using a commercial kit (BioVision, Mountain View, CA) by following the manufacturer’s protocol, with slight modifications (28).

**NF-κB Activation**—NF-κB activation was measured with a Trans-AM NFκB kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. In this assay, the oligonucleotide containing the NFκB consensus sequence (5’-GAGGACTTCC-3’) is immobilized onto a 96-well plate. Only the active form of NFκB in the cell extract specifically binds to this oligonucleotide. Epitopes on p50, p52, p65, p-re1, and p16B are accessible only when NFκB is bound to its target DNA. Nuclear extracts containing 200 μg were added to the wells, followed by the addition of a primary antibody against p65 and the horseradish peroxidase-conjugated secondary antibody. The optical density was determined on an absorbance plate reader at 450 nm.

**Assessment of Cell Death**—Apoptotic and necrotic cell death was assessed independently. At the indicated time points, floating cells were collected, and adherent cells were trypsinized, centrifuged, and resuspended in FBS (10%) containing 200 μM digitonin (Calbiochem) and 50 μM propidium iodide (Molecular Probes). Digitonin permeabilizes the plasma membrane and allows propidium iodide to enter cells and stain all nuclei. Staining nuclei with propidium iodide in the presence of digitonin is analogous to staining nuclei with cell-permeable dyes such as 4',6-diamidino-2-phenylindole. Fragmented nuclei were scored as apoptotic and counted using a 40× microscope objective and a rhodamine barrier filter set. Necrotic cell death was assessed by incubating cells with trypan blue. Trypan blue enters cells and stains nuclei when the plasma membrane permeability barrier fails, a hallmark of necrosis (29).

Round, trypan blue-stained nuclei were counted as necrotic. At least 100 cells were counted from three different microscopic fields for each sample. Results were expressed as the percentage of all cells that were apoptotic or necrotic.

Additionally, apoptotic cell death was assessed with a Cell Death Detection ELISA Plus kit (Roche Applied Science, Indianapolis, IN), according to the manufacturer’s instructions. Endogenous endonucleases activated during apoptosis cleave double-stranded DNA to mono- and oligonucleosomes of 180-bp multiples, which the ELISA kit measures. DNA fragments were measured in the cytoplasmic fractions.

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

**RESULTS**

**NAC Enhances Hypoxia-induced Apoptosis**—NAC is a nontoxic precursor of GSH and has been reported to protect cells against oxidative stress (22). We exposed p53−/− MEFs to hypoxia for 12 h in the presence and absence of NAC. Hypoxia alone caused 12% apoptosis compared with 2% during normoxia, as assessed by nuclear morphology typical of apoptosis (Fig. 1A). Apoptotic cells showed fragmented nuclei when stained with propidium iodide in the presence of digitonin (Fig. 1A). Treatment with NAC enhanced hypoxia-induced apoptosis in a dose- and time-dependent manner (Fig. 1, A and B). Apoptosis increased from 12% without NAC to 46% with 20 mM NAC (Fig. 1A). During normoxia, NAC increased apoptosis from 2.7% without NAC to 4.2% with 20 mM NAC (Fig. 1A). However, this increase of apoptosis was not dose-dependent. In contrast to p53−/− MEFs, p53−/− MEFs were completely resistant to hypoxia and NAC (data not shown). In p53−/− MEFs, NAC promoted hypoxia-induced apoptosis in a time-dependent manner. Apoptosis increased from 1.6, 2.8, 5.7, and 12.0% without NAC to 1.9, 7.9, 19.5, and 46.0% with 20 mM NAC after 0, 4, 7, and 12 h of hypoxia, respectively (Fig. 1B). In the subsequent experiments, we chose 20 mM NAC to characterize the cellular and molecular targets of NAC. This concentration was chosen because it maximally promoted apoptosis during hypoxia (Fig. 1A and data not shown). Furthermore, previous studies show that 20 to 30 mM NAC inhibits ROS formation and NFκB activation in cancer cells (19, 30).

To further confirm that hypoxia-induced and hypoxia plus NAC-induced cell death was primarily apoptotic, histone-associated DNA fragments were measured in cytoplasmic fractions. Hypoxia alone increased DNA fragments 20% compared with normoxia, whereas hypoxia plus NAC increased DNA fragments by 260% compared with normoxia (Fig. 1C). During normoxia, however, NAC had no effect on DNA fragment formation.

To assess necrotic cell death, MEFs were incubated with 0.2% trypan blue. Round nuclei stained with trypan blue were scored as necrotic. During normoxia, 11% of cells were necrotic (Fig. 1D). Hypoxia, hypoxia plus NAC, and NAC during normoxia did not increase necrosis significantly. The small percentage of necrosis observed in normoxic cells may be partly attributable to trypan blue staining and not related to experimental treatments. Together, our results indicate that cell death promoted by NAC was apoptotic.

We also determined the effect of NAC on two human cancer cell lines. MIA PaCa-2 pancreatic cancer cells were exposed to hypoxia in the presence and absence of 20 mM NAC. NAC increased apoptosis from 9% without NAC to 30% with 20 mM NAC after 15 h of hypoxia (Fig. 1E). In A549 lung carcinoma cells, NAC increased apoptosis from 6% without NAC to 22% with 20 mM NAC after 26 h of hypoxia (Fig. 1F). NAC alone during normoxia caused a small increase of apoptosis in A549 cells but no increase in MIA PaCa-2 cells.

**NAC Does Not Accelerate Hypoxia-induced Cytochrome c Release**—Next, we assessed whether NAC-enhanced apoptosis during hypoxia was mediated by accelerated cytochrome c release from mitochondria to the cytosol. After 12 h of exposure to hypoxia, a portion of cytochrome c was released from mitochondria to the cytosol (Fig. 2A). However, NAC did not change the extent of cytochrome c release, indicating that NAC-mediated enhancement of apoptosis acted downstream of mitochondria.

Because NAC did not seem to enhance apoptosis through mitochondrial cytochrome c release, we determined whether post-mitochondrial events, such as PARP cleavage, were affected by NAC during hypoxia. PARP is a target for cysteine proteases, and 116-kDa PARP is frequently cleaved by caspase-3 into an 85-kDa fragment during apoptosis (31). After 7 and 14 h of hypoxia alone, we could not detect the cleaved 85-kDa fragment of PARP by Western blot analysis (Fig. 2B). In contrast, hypoxia in combination with NAC produced the 85-kDa PARP-degraded product (Fig. 2B). Collectively, the results indicate that enhancement of hypoxia-induced apoptotic cell death by NAC was not mediated by increased cytochrome c release but rather through some other downstream effector molecules that resulted in PARP cleavage.

**NAC Blocks Hypoxia-induced ROS Formation**—NAC is usually associated with protection against ROS-dependent cell killing (32). Here, however, NAC enhanced hypoxia-induced apoptosis (Fig. 1). Accordingly, we assessed the effect of NAC on ROS generation during hypoxia. ROS were detected with CM-H2DCFDA, which becomes highly fluorescent DCF when oxidized by ROS (13). Exposure of MEFs to 4 h of hypoxia increased DCF fluorescence by 65% compared with normoxia (Fig. 3A). NAC almost completely abolished hypoxia-induced DCF fluorescence, indicating that NAC blocked hypoxia-induced ROS formation. We also tested the effect of other anti-
oxidants on hypoxia-induced ROS generation. Trolox, a vitamin E analog (1 mM), and polyphenolic antioxidant epigallocatechin-3-gallate (EGCG) (100 μM) also prevented ROS formation during hypoxia (Fig. 3A). Trolox was even better at scavenging ROS than NAC. However, unlike NAC, neither trolox nor EGCG enhanced hypoxia-induced apoptosis (Fig. 3B). The results indicate that NAC-mediated enhancement of apoptosis was not a direct consequence of inhibition of ROS formation.

In some systems, ROS activate the MAPK (p44/42) pathway (33). To determine whether NAC acts through MAPK, we assessed the effect of NAC on MAPK activation. In MEFs transformed with Ras and E1A, the MAPK pathway is constitutively active, and therefore we observed some phosphorylation of p44/42, as shown in Fig. 3C. After 4 h of hypoxia, however, phospho-p44/42 protein levels increased by 4.8-fold compared with normoxia (Fig. 3C). NAC caused only a slight decrease in p44/42 phosphorylation, whereas a specific inhibitor of the MAPK (PD98059, 50 μM) virtually abolished phosphorylation of p44/42. These findings are consistent with the conclusion that the pro-apoptotic effect of NAC was not attributable to MAPK activation.

Glutathione Is Involved in Hypoxia-induced Apoptosis by NAC—NAC is rapidly taken up by cells. Inside cells, endogenous esterases release free L-cysteine, which is a precursor for GSH synthesis (20). To confirm that NAC is capable of increasing intracellular GSH levels, we measured intracellular GSH with high-performance liquid chromatography. NAC increased GSH in normoxic and hypoxic cells (Fig. 4A). After 4 h of exposure to hypoxia, NAC-treated cells had higher GSH compared with untreated cells (Fig. 4A).

The results of Figs. 1 to 4 suggest that NAC-mediated enhancement of apoptosis in hypoxic cells may be attributable to increased intracellular GSH. To increase cellular GSH directly...
N-Acetyl-l-cysteine Potentiates Hypoxia-induced Apoptosis

NAC does not accelerate hypoxia-induced cytochrome c release. A, MEFs were pre-incubated with 0 and 20 mM NAC as indicated and exposed to hypoxia and normoxia for 12 h. Mitochondrial and cytosolic fractions were prepared as described in “Materials and Methods” and subjected to Western blot analysis using anti-cytochrome c (Cyt c) and F10α-ATPase (mitochondrial marker) antibodies, respectively. B, MEFs were pre-treated with 20 mM NAC for 1 h, followed by hypoxia for 7 and 14 h. Whole-cell lysates were subjected to Western blot analysis to detect PARP cleavage. Actin was used as a loading control.

without increasing l-cysteine, we treated cells with GSH monoester (4 mM). Similar to NAC, GSH monoester passes freely through the plasma membrane and is cleaved by endogenous esterases. The result is an increase of GSH. Incubation of cells with GSH monoester for 4 h increased GSH levels during normoxia and hypoxia as expected (Fig. 4A). GSH monoester treatment also enhanced hypoxia-induced apoptosis from 13 to 30% after 12 h exposure to hypoxia (Fig. 4B). Although GSH monoester increased GSH to a greater extent than NAC, GSH monoester promoted apoptosis less effectively than NAC. The reason for this discrepancy was probably the difference in incubation time between the GSH analysis and apoptosis assay (4 h versus 12 h). GSH monoester increases GSH more rapidly than NAC, whereas NAC sustains high GSH levels for a longer period. Furthermore, to address more directly whether increased apoptosis with NAC was attributable to an increase in total cellular thiol or attributable to a more specific increase in GSH, we treated hypoxic cells with the d-isomer of NAC, which is not a precursor of GSH synthesis, and dithiothreitol (DTT), which is a general thiol reductant. However, neither d-NAC nor DTT enhanced hypoxic apoptosis (Fig. 4C). Collectively, these results were consistent with the conclusion that GSH rather than l-cysteine per se was responsible for enhancing hypoxic apoptosis.

Cellular acidosis is usually associated with hypoxia in solid tumors. To exclude the possibility that a decrease of pH was responsible for enhanced apoptosis induced by NAC and GSH monoester, we measured the pH of the medium in the presence and absence of NAC during normoxia and hypoxia. Exposure to 12 h of hypoxia decreased medium pH by 0.1 pH unit compared with normoxia. NAC and d-NAC further decreased the medium pH by 0.3 units compared with hypoxia alone. However, only NAC promoted hypoxia-induced apoptosis (Fig. 4C). GSH monoester did not change pH during hypoxia but did promote apoptosis during hypoxia (Fig. 4B). Therefore, we conclude that enhanced hypoxic apoptosis by NAC and GSH monoester was not attributable to decreased pH but rather attributable to increased GSH. These results are in agreement with the recent study of human RKO colorectal carcinoma cells, where changes in medium pH between 7.3 and 6.9 had no effect on apoptosis (30).

NAC Does Not Prevent Hypoxia-induced IkBa Degradation and NFkB Translocation to the Nucleus—Because NAC did not block activation of the MAPK survival pathway (Fig. 3C), we turned our attention to the redox-sensitive transcription factor NFkB, which is activated by numerous factors, including ROS (8). NFkB activation is negatively regulated by a family of IkB repressor proteins, of which the most extensively studied is IkBa. Ubiquitin-mediated proteolytic degradation of IkBa leads to the translocation of NFkB from the cytoplasm to the nucleus, where it activates transcription. To determine the role, if any, of NFkB signaling in NAC-mediated promotion of apoptosis, we determined the effect of NAC on IkBa protein levels. MEFs were exposed to hypoxia for 4 h in the presence and absence of NAC. Cytosolic fractions were prepared, and IkBa proteins were analyzed by Western blotting. As expected, hypoxia induced IkBa protein degradation (Fig. 5A). Surprisingly, NAC failed to prevent IkBa protein degradation. To confirm that IkBa protein degradation after hypoxia was mediated by proteasomes, we measured IkBa protein levels with and without 20 μM o-methylomuralide, a cell-permeable, irreversible inhibitor of proteasome function (34). Methylomuralide equally inhibited IkBa degradation during hypoxia and hypoxia plus NAC (Fig. 5A).

Immunocytochemical analysis of the p65 subunit of NFkB was also performed after 4 h hypoxia in the presence and absence of NAC. During normoxia, p65 was primarily localized to the cytoplasm, and large dark voids in the confocal image corresponded to the Hoechst-stained nuclei (Fig. 5B). These findings indicated that NFkB was localized to the cytoplasm and therefore was not activated. During hypoxia, p65 became localized to the nuclei, showing directly NFkB protein levels with and without 20 μM o-methylomuralide, a cell-permeable, irreversible inhibitor of proteasome function (34). Methylomuralide equally inhibited IkBa degradation during hypoxia and hypoxia plus NAC (Fig. 5A).

NAC Inhibits Hypoxia-induced Transactivation of NFkB—Although hypoxia induced NFkB nuclear translocation, transcriptional activation of nuclear NFkB may be blocked by other factors. Therefore, a gene reporter assay for NFkB was performed. Hypoxia increased NFkB luciferase reporter activity by 150% compared with normoxic activity (Fig. 6A). NAC prevented the increase of reporter activity completely (Fig. 6A). We next determined whether increased transcriptional activation of NFkB was attributable to increased p65-NFkB binding to DNA, as assessed by a p65-NFkB-specific ELISA using nuclear extracts (Fig. 6B). In this approach, the NFkB response element DNA was attached to microtiter plate wells, and nuclear NFkB capable of binding this sequence was measured by ELISA. Hypoxia alone (4 h) increased p65-NFkB DNA binding compared with normoxia. NAC decreased hypoxia-induced p65-NFkB DNA binding (Fig. 6B). By contrast, the non-thiol antioxidants trolox and ECGD did not reverse hypoxia-induced increase of p65-NFkB DNA binding (data not shown). Taken together, these results indicate that hypoxia activated NFkB translocation to the nucleus and enhanced NFkB binding to DNA, resulting in activation of NFkB-dependent gene expression. NAC treatment during hypoxia did not abolish NFkB.
nuclear translocation but blocked DNA binding and subsequent gene activation.

**NAC Inhibits Hypoxia-induced XIAP Protein Expression—**

NFκB transcriptionally activates several anti-apoptotic target genes including Bcl-xL, Bcl-2, c-FLIP, XIAP, and cellular inhibitors of apoptosis (c-IAPs) (7). XIAP is of particular interest, because XIAP blocks apoptosis at a post-mitochondrial level by inhibiting caspase-9. Exposure of MEFs to 4 h of hypoxia increased protein expression of XIAP, an effect down-regulated by NAC (Fig. 7A). XIAP contains a caspase-binding domain and becomes bound to activated caspase-9, thus blocking caspase-3 activation and apoptosis (35). Exposure of oncogenic MEFs to 7 h of hypoxia did not increase caspase-3 activity compared with normoxia, suggesting that the hypoxia-driven increase in XIAP protein expression may interfere with caspase-3 activation (Fig. 7C). Exposure of oncogenic MEFs to 7 h of hypoxia did not increase caspase-3 activity compared with normoxia, suggesting that the hypoxia-driven increase in XIAP protein expression may interfere with caspase-3 activation (Fig. 7C). Hypoxia in the presence of NAC, by contrast, led to a 467% increase in caspase-3 activity (Fig. 7C), which paralleled apoptotic cell death (Fig. 1B). The effect of hypoxia on other anti-apoptotic genes that are transcriptionally activated by NFκB was also determined. However, no change in the protein expression of Bcl-xL, Bcl-2, or c-FLIP was observed during hypoxia (Fig. 7B). Also, hypoxia did not alter the protein status of Bax (Fig. 7B). Taken together, we identified XIAP as a target gene for NFκB activation during hypoxia.

**DISCUSSION**

The main finding of the present study was that NAC increased hypoxic apoptosis in MEFs, Mia PaCa-2 human pancreatic cancer cells, and A549 human lung carcinoma cells (Fig. 1). Increased apoptosis was not attributable to decreased ROS formation because other antioxidants that suppressed ROS did not increase apoptosis (Fig. 3). Rather, NAC appeared to regulate a transcriptional checkpoint in the NFκB signaling pathway. NAC did not inhibit NFκB translocation to the nucleus (Fig. 5). However, NAC interfered with NFκB-DNA binding and blocked hypoxia-induced NFκB transcriptional activation (Fig. 6). As a consequence, a NFκB-dependent XIAP expression was suppressed, which accounted, at least in part, for increased caspase-3 activation and enhanced apoptosis during hypoxia (Fig. 7).

Cellular sensors for hypoxia include hypoxia inducible factor-1, activator protein-1, and vascular endothelial growth factor, which are activated in response to hypoxia to allow cells to survive a hypoxic environment (36). Therefore, cancer cells are usually resistant against hypoxia (30, 37), in agreement with the results shown in this study (Fig. 1). Because ROS are formed during hypoxia (Fig. 2A), ROS may be required for activation of these survival genes (21). Hypoxia (1 to 5% O2) increases mitochondrial ROS production (14), and mitochondrial ROS may initiate signaling cascades such as NFκB and MAPK to protect from cell death (38, 39). By contrast, decreases in intracellular ROS formation during hypoxia may allow expression of genes that are otherwise suppressed by ROS (40). Here we show that NAC increases hypoxia-induced apoptosis (Fig. 1). Our results agree with earlier reports that antioxidants such as NAC increase UV radiation-induced apoptosis in C8161 melanoma cells (41). However, NAC-mediated
enhancement of apoptosis in hypoxic MEFs was not attributable to inhibition of p42/44 MAPK phosphorylation, as was the case for C8161 melanoma cells after UV radiation (41). Although hypoxia increased MAPK phosphorylation, a measure of MAPK activation, NAC did not prevent this phosphorylation (Fig. 3C).

To determine whether NAC-mediated enhancement of apoptosis was attributable to inhibition of ROS, we examined the effects of the antioxidants trolox and EGCG on ROS and cell viability. Similar to NAC, trolox and EGCG blocked hypoxia-induced ROS formation (Fig. 3A). Surprisingly, neither trolox nor EGCG enhanced hypoxic apoptosis (Fig. 3B). Therefore, NAC-mediated enhancement of hypoxic apoptosis did not appear to be the consequence of inhibition of ROS formation. Trolox is a vitamin E analog, whereas EGCG is a polyphenolic antioxidant. Neither is a precursor of GSH. Therefore, we hypothesized that NAC-mediated enhancement of apoptosis occurred via increased intracellular GSH. Accordingly, we measured intracellular GSH levels after treatment with NAC and GSH monoester, which also increases GSH. Both NAC and GSH monoester increased GSH during normoxia and hypoxia. After 4 h of treatment, GSH monoester loads GSH into cells more rapidly than NAC, as measured after 4 h (Fig. 4A). The differences between NAC and GSH monoester may relate to the time course of GSH supplementation by the two agents. GSH monoester loads GSH into cells more rapidly than NAC, as measured after 4 h (Fig. 4A), whereas NAC as a GSH precursor likely sustains high GSH levels over a longer time, up to 12 h when apoptosis was assessed. Thus, although both NAC and GSH monoester enhanced hypoxic apoptosis, GSH monoester appeared to promote hypoxic apoptosis somewhat less than NAC (Fig. 4B), because increased GSH levels after GSH monoester treatment likely declined more rapidly than after NAC treatment.

Further evidence that enhanced apoptosis is a consequence of increased GSH comes from the data that neither d-NAC nor DTT enhanced hypoxic apoptosis (Fig. 4C). d-NAC loads cells with d-cysteine and increases total thiol content, but d-cysteine cannot be used for GSH synthesis (42). Similarly, DTT promotes thiol reduction but does not increase GSH. Taken together, the present data indicate that NAC-mediated enhancement of hypoxic apoptosis was not a direct consequence of ROS inhibition but rather depended on GSH status.

ROS can cause direct cellular damage and can also act as second messengers for signal transduction pathways. In particular, NFκB is a redox-regulated transcription factor that is important in signaling and in the development and progression of malignancies (7). The involvement of ROS in the activation of NFκB is based on the observations that agents that stimulate ROS production activate NFκB, whereas antioxidant compounds inhibit NFκB activation (43–45). Another study suggests that ROS do not directly mediate NFκB activation. The study proposes that NAC blocks tumor necrosis factor-α-stimulated NFκB activation by inhibiting the binding of tumor necrosis factor-α to its receptors without affecting interleukin-1- or 12-O-tetra-
decanoylphorbol-13-acetate-stimulated NFκB activation, and that endogenous ROS from the Rac/NADPH oxidase system do not mediate NFκB activation (19). Accordingly, we investigated whether NAC-mediated enhancement of hypoxic apoptosis was attributable to inhibition of NFκB.

The activation of NFκB is controlled by a family of IκB repressor proteins (11). A critical step in NFκB activation is IκB phosphorylation by kinases such as IKK, which directs IκB into 26 S proteasomes for degradation. Exposure to hypoxia for 4 h induced IκB degradation, a step toward NFκB activation (Fig. 5A). Although NAC blocked ROS formation, NAC did not prevent IκB degradation. This finding indicated that ROS were not essential for IκB inactivation and that NAC was not acting at this step in the NFκB cascade.

Proteasome inhibition blocked IκB degradation during hypoxia (Fig. 5A). Ubiquitin-conjugating enzymes are inhibited by high levels of oxidized GSH (45). In airway epithelial cells exposed to H₂O₂, inhibition of ubiquitin-dependent proteolysis results in sustained IκB-dependent suppression of NFκB and increased apoptosis (46). However, IκB degradation during hypoxia to MEFs in the present work was not inhibited by NAC (Fig. 5A), which implies that increased GSH after NAC was not affecting the efficiency of IκB proteolysis. In some systems, tyrosine 42 phosphorylation of IκBα suppresses IκBα-dependent NFκB inhibition, an effect not mediated by proteasomal degradation of IκB (6). This was not the case for MEFs during hypoxia, because IκBα was degraded both in the presence and absence of NAC (Fig. 5A). Another mechanism of IκB inactivation is glutathione-dependent protein S-thiolation (47) of cysteine 179 in IKKα and IKKβ (39). However, such mechanism does not account for NAC-dependent NFκB inactivation during hypoxia, because the redox-sensitive site in the present study was downstream of IκBα degradation and subsequent p65 nuclear translocation (Fig. 5B).

During hypoxia, NFκB translocated to the nucleus. NAC did not prevent this translocation (Fig. 5B). In the nucleus, NFκB binding to promoter regions of NFκB target genes depends on conserved cysteines. The redox state of these cysteines is maintained by the thioredoxin/thioredoxin reductase system (48). However, the GSH precursor NAC decreased rather than increased DNA binding by NFκB (Fig. 5B). These considerations argue against involvement of thioredoxin in hypoxia-induced NFκB activation, but further studies will be needed to identify the molecular target(s) of NAC-mediated blockade of NFκB activation during hypoxia.

Hyoxia increased XIAP protein expression, which was down-regulated by NAC (Fig. 7A). The hypoxia-dependent up-regulation was specific for XIAP, because the other NFκB target genes, including Bcl-xL, Bcl-2, Bax, and c-FLIP, remained unchanged during hypoxia (Fig. 7B). A similar effect of hypoxia on Bcl-xL, Bcl-2, and Bax has been reported earlier in malignant glioma cells (49). XIAP suppresses apoptosis post-mitochondrially by inhibiting cytochrome c-dependent activation of caspase-9 and caspase-3. NAC, by decreasing XIAP expression, may enhance caspase activation after cytochrome c release. Consistent with this view is that NAC did not alter cytochrome c release during hypoxia (Fig. 2A) but did increase caspase-3 activation (Fig. 7C).
p53-deficient MEFs were virtually completely resistant to apoptosis after hypoxia and hypoxia plus NAC (data not shown), whereas hypoxia induced cytochrome c release and apoptosis in p53+/− MEFs, especially in the presence of NAC (Fig. 2A). Hypoxia also increased p53 protein expression in p53+/− MEFs. Thus, hypoxia activated a p53-dependent intrinsic pathway of apoptosis. The initial activation of this intrinsic pathway was not sensitive to NAC, because NAC did not alter cytochrome c release during hypoxia. Possible mechanisms of p53-dependent induction of the intrinsic apoptotic pathway involve increased expression of pro-apoptotic genes of the Bcl-2 family, such as Bax, Noxa, and PUMA (50–52). p53 can also directly activate Bax and trigger cytochrome c release (50). Although we did not observe an increase of total Bax protein during hypoxia (Fig. 7B), p53 protein may activate Bax translocation to mitochondria and therefore cause cytochrome c release in a p53-dependent manner (50). Two other pro-apoptotic proteins, PUMA and Noxa, are also induced in response to p53 and subsequently translocate to mitochondria to trigger cytochrome c release (51, 52). Further studies are required to define the roles these proteins play in p53-dependent cytochrome c release during hypoxic apoptosis.

In conclusion, activation of NFκB-dependent survival pathways confers death resistance during hypoxia, a common feature of most solid tumors. Intracellular GSH supplementation with agents such as NAC and GSH monoester block this activation by preventing NFκB from binding to and activating the promoter regions of its target genes. Thus, manipulations to increase cellular GSH levels could potentially be used therapeutically to sensitize cancer cells to hypoxic apoptosis. Down-regulation of XIAP, an NFκB target gene, might also complement traditional anticancer therapy by selectively enhancing hypoxic apoptosis at a post-mitochondrial level (Fig. 8).

Acknowledgments—We thank Dr. Luke Sweda for GSH measurements, Dr. Santanu Bose for NFκB-luc plasmid, Dr. Ian Cotgreave for D-NAC, and Dr. Scott Lowe for the MEF cell line. We also thank 2 S. Qanungo, M. Wang, and A.-L. Nieminen, unpublished results.
REFERENCES

1. Helmlinger, G., Yuan, F., Delian, M., and Jain, R. K. (1997) *Nat. Med.* 3, 177–182.
2. Schmaltz, C., Hardenbergh, P. H., Wells, A., and Fisher, D. E. (1998) *Mol. Cell. Biol.* 18, 2844–2854.
3. Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., Lowe, S. W., and Reed, J. C. (1999) *Science* 286, 1358–1366.
4. Lin, A., and Karin, M. (2003) *Cell Biol.* 7, 3673–3682.
5. Greten, F. R., and Karin, M. (2004) *Cancer Lett.* 206, 193–199.
6. Fan, C., Li, Q., Ross, D., and Engelhardt, J. F. (2003) *J. Biol. Chem.* 278, 20732–20739.
7. Lin, A., and Karin, M. (1999) *Sci. STKE.* 1999, RE1.
8. Rothwarf, D. M., and Karin, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 100, 10890–10895.
9. Wang, C. Y., Guttridge, D. C., Mayo, M. W., and Baldwin, A. S., Jr. (1999) *Mol. Cell. Biol.* 19, 5923–5929.
10. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 224, 4235–4242.
11. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
12. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
13. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
14. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
15. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
16. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
17. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
18. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
19. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
20. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
21. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
22. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
23. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
24. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
25. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
26. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
27. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
28. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
29. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
30. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
31. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
32. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
33. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
34. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
35. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
36. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
37. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
38. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
39. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
40. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
41. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
42. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
43. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
44. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
45. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
46. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
47. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
48. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
49. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.
50. Wang, G. L., Jiang, B. H., and Semenza, G. L. (1995) *Biochem. Biophys. Res. Commun.* 212, 550–556.