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Opposing Regulation of Choline Deficiency-induced Apoptosis by p53 and Nuclear Factor κB*

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We have previously shown that fetal rat brain cells, preneuronal (PC12), and hepatocyte (CWSV-1) cells undergo apoptosis during choline deficiency (CD). The PC12 and epithelial cell culture models were used to determine the molecular mechanism by which CD induces apoptosis. Our data indicate that CD leads to both growth arrest and apoptosis in a subpopulation of cells, which correlate with the up-regulation of the tumor suppressor protein p53 and concurrent up-regulation of the cyclin-dependent kinase-inhibitor $p21^{WAF1/CIP1}$. Additionally, CD induced both a G_1/S and a G_2/M arrest. Transient transfection of a dominant negative p53 (p53DN) construct into PC12 cells, which inhibited endogenous p53 activation, significantly reduced the induction of apoptosis associated with CD. Interestingly, CD also induced the persistent activation of the transcription factor NF-κB. Activation of NF-κB has been shown to promote cell survival and proposed to antagonize p53. Consistent with this, expression of a super-repressor form of IκBα (SR-IκBα) that functions to strongly inhibit NF-κB activation, profoundly enhanced cell death during CD. In summary, these results suggest that the effects of CD on apoptosis and subsequent cell survival are mediated through two different signaling pathways, p53 and NF-κB, respectively. Taken together, our data demonstrates the induction of opposing mechanisms associated with nutrient deficiency that may provide a molecular mechanism by which CD promotes carcinogenesis.

Choline is an essential nutrient required for normal function of all cells (1–3). Choline is essential for DNA methylation and DNA synthesis in cells (1, 3). However, long term dietary deficiency of choline (CD)[1] has been associated with increased incidence of tumors in various organs of rats (4). Consistent with this point, short term CD has been associated with elevated expression of mRNAs for several proto-oncogenes including c-*myc* (5), c-Ha-*ras* (6), and c-*fos* (7). Other pathophysiologic features associated with CD include caspase activation with diminished membrane phospholipids (8), induction of cyclooxygenase 2 (Cox-2) (9), elevated serum TNF-α (10), increased capability to transform rat hepatocytes (11), increased protein kinase C activity (12), enhanced induction of hepatocellular carcinoma in animals (13), and increased oxidative DNA damage (14). More recently, we have shown that CD can inhibit cell proliferation and can induce apoptosis *in vivo* and *in vitro* (15, 16). Although CD is associated with many pathological effects, the molecular mechanism(s) by which CD exerts these effects has not yet been elucidated.

Apoptosis is a physiological cell death mechanism that occurs during development, in response to cell damage, and in response to external stress (17). The induction of apoptosis is a multi-step mechanism that includes tightly regulated signal transduction pathways involving protease activation. In several cases, the induction of apoptosis is associated with activation of p53, a transcription factor sensitive to cellular stress (18, 19). Additionally, p53 functions to induce growth arrest in part by activating the expression of $p21^{WAF1/CIP1}$ (19, 20), a cyclin-dependent kinase inhibitor (CdkI) (21). $p21^{WAF1/CIP1}$ is a key molecule that regulates the cellular response to DNA damage and to inhibition of the protein kinases that drive the cell cycle (22). Increased expression of $p21^{WAF1/CIP1}$ by p53 transcription correlates with cell cycle control by the induction of a G_1 and/or a G_2/M arrest or apoptosis (19, 20).

Another key transcription factor involved in the control of apoptosis is nuclear factor κB (NF-κB) (23–25). Following induction of nuclear translocation by exposure of cells to stimuli including inflammatory mediators, NF-κB regulates the expression of genes encoding cytokines, cytokine receptors, and cell adhesion molecules (23–25). This control of inflammatory gene expression likely explains the association of NF-κB activation with inflammatory diseases such as arthritis (26) and inflammatory bowel disease (27). The first evidence for a role NF-κB as a regulator of apoptosis was through studies showing that knockout of the p65/RelA subunit of NF-κB led to extensive liver apoptosis during development (28). Subsequently, NF-κB was shown to be an inhibitor of TNF-induced apoptosis (29) and to block apoptosis induced by other stimuli including oncoprotein expression (30) and stress inducers such as chem-

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[1] The abbreviations used are: CD, choline deficiency; NF-κB, nuclear factor κB; TNF, tumor necrosis factor; γ-IRR, γ irradiation; TUNEL, Tdt-mediated dUTP nick end labeling; ELISA, enzyme-linked immunosorbent assay; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; SR-IκBα, super-repressor IκBα; β-gal; β-galactosidase; EMSA, electrophoretic mobility shift assay; ANOVA, analysis of variance; CS, choline sufficient; wt, wild type; mut, mutant; ts, temperature-sensitive; p53DN, p53 dominant negative.

otherapy (31). More recently the activation of NF-κB was shown to be associated with the regulation of cell growth through the induction of genes such as c-*myc* (32) and cyclin D1 (33). Consistent with its involvement in cell growth, NF-κB activation is required for the induction of oncogenic transformation and tumor formation in certain models of cancer (30, 34, 35). At least one mechanism whereby NF-κB facilitates oncogene-driven transformation is through the suppression of apoptosis (30).

While many factors involved in regulation of apoptosis induced by TNF receptor family signals have been elucidated, details of nutrient regulation of apoptotic signaling are few. In particular, the molecular mechanism(s) for CD-induced apoptosis and its associated carcinogenic potential have not been defined. Because p53 and NF-κB are transcriptional regulators associated with cellular stress and oncogenic events and are antagonistic in many cell systems (36, 48), we asked whether these transcription factors are involved in CD-induced apoptosis. In this study, our findings may provide a molecular mechanism for cell survival and the further development of hepatocellular carcinoma associated with CD.

MATERIALS AND METHODS

Cell Culture—PC12 cells were cultured as previously described (15). Briefly, cells were plated at a density of 2 × 105 cells/plate in Dulbecco's modified Eagle's medium/F12 choline-free media (American Biogenics) supplemented with 10% fetal bovine serum, 5% horse serum, and 1% penicillin-streptomycin and maintained at 37 °C in a 5% humidified incubator. For the following experiments, cells at 70% confluency were maintained in defined Dulbecco's modified Eagle's medium/F12 serum-free media as previously indicated (15). A parallel group of cells grown under the same conditions were exposed to either 0 rads (Sham) or 500 rads γ irradiation (γ-IRR) (100 rads = 1 Gy). Cell growth was assessed by trypan blue dye exclusion for number of viable cells. Three independent experiments were completed in duplicate. The H1299 cell line, which contains a homozygous deletion of the p53 gene (37), was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and G418/ml (Life Technologies, Inc.). This allowed selection for stable clones expressing the temperature-sensitive p53 (H1299-tsp53). The H1299 and H1299ts-p53 were kind gifts of Dr. N. Raab-Traub (University of North Carolina, Chapel Hill, NC) and have been described previously (38).

Western Blot Analysis—Western blotting was performed as previously described (39). Briefly, equal amounts of cytoplasmic extracts (60 μg) were loaded and separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were blocked with 5% milk proteins in 1× Tris-buffered saline and 0.5% Tween 20 buffer and probed with either a specific monoclonal anti-mouse p53 antibody (1:1000, NovaCastra Labs) or a polyclonal anti-rabbit p21 antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Blots were probed with a secondary anti-mouse IgG (p53 detection) or anti-rabbit IgG (p21 detection) conjugated with horseradish peroxidase (1:10,000). Proteins were visualized using the enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech) and audioradiography.

Apoptosis Assays: TUNEL Assay—PC12 cells were cultured with and without choline as described. After 48 h apoptotic cells were detected by Tdt-mediated dUTP nick end labeling (TUNEL) using the cell death *in situ* assay (Roche Molecular Biochemicals). The H1299-tsp53 cells were cultured with and without choline for 48 h either at 32 °C to induce the p53 wild type (wt-p53) conformation or at 39 °C for the p53 mutant (mut-p53) conformation. All cells were counterstained with fluorescein mounting medium (DAB, Oncor) and viewed by fluorescent microscopy.

DNA Fragmentation Assay—The H1299-tsp53 cells were cultured as described above. After 48 h cell death was detected by the ELISA apoptosis assay (Roche Molecular Biochemicals) as described previously (39), and the percentage of apoptosis was quantitated. Staurosporine was used as a positive control for the induction of apoptosis. Samples were run in triplicate, and data represents three independent experiments.

Cell Cycle Analysis—Flow cytometric analysis was performed as previously described (40). Briefly, PC12 cells were incubated with and without choline. After 48 h, log-phase cultures were incubated at 37 °C for 4 h in 10 mM bromodeoxyuridine (BrdUrd). Cells were washed with PBS, trypsinized and fixed in ice-cold 95% ethanol/5% PBS. Fixed cells were incubated in 0.08% pepsin for 20 min at 37 °C, followed by 2 N HCl incubation for 20 min at 37 °C. Cells were neutralized, resuspended in incomplete Freund's adjuvant + 0.5% Tween 20, and then stained with α-BrdUrd-fluorescein isothiocyanate (Becton-Dickinson, San Jose, CA) in the dark for 30 min. Stained nuclei were resuspended in 5 μg/ml RNase A and 50 μg/ml propidium iodide (Sigma), incubated at 37 °C for 15 min followed by an additional 15 min in the dark on ice. Cell cycle analysis was performed on a FAC Scan instrument (Becton-Dickinson) at 488 nm. Data were analyzed by Modfit computer program, and data are representative of three independent experiments.

Transient Transfection and β-Galactosidase (β-Gal) in Situ Assay—PC12 cells were co-transfected using LipofectAMINE reagent/ml (Life Technologies, Inc.) with either the empty expression vector (pDCR), the expression plasmid that contained the X-gal binding site (pCMV-lacZ), with the mutant $p53^{135}$ expression vector that acted as a dominant negative inhibitor of endogenous p53 (41) (p53DN; gifts from Dr. M. Mayo, Univ. of North Carolina-Chapel Hill, NC) (29, 39), or with the 3XκB-luc reporter. Transfections used 5.0 μg of the pDCR empty vector alone or 1.0 μg of either the pCMVlacZ, the $p53^{135}$ expression vectors, or the 3XκB-luc reporter and were brought to a final concentration of 5.0 μg with the empty vector for 24 h. After 24 h, cells were treated with or without choline. A parallel transfection experiment included the use of the super-repressor IκBα (SR-IκBα) protein that binds endogenous IκBα preventing NF-κB nuclear translocation. PC12 cells were co-transfected with either 1.0 μg of DNA from either the empty expression vector (pDCR), the $p53^{135}$ expression vector (p53DN), or the SR-IκBα expression vector and brought to a final concentration of 5.0 μg with the empty vector. In general, 0.2×10^6 PC12 cells were plated in 24-well plates and grown overnight before transfection. Fresh media was added 1–2 h prior to transfection. LipofectAMINE-DNA complexes were allowed to form for 30 min in serum-free media before adding washed cells. Cells were incubated with complexes for 7 to 8 h followed by a media wash and then incubated for 24 h. The β-gal *in situ* assay was used to detect transfected cells as a measure of transfection efficiency and correspondingly to cell survival (39). Briefly, culture plates were washed in 2 ml PBS, fixed in formaldehyde for 5 min at room temperature, washed twice in PBS, and then incubated with X-gal dye in PBS-cyanide solution overnight at 37 °C. β-Gal activity was assessed by counting the number of viable β-gal-expressing cells stained blue in relation to total cells. Data are presented as a percent of control.

Cell Viability Assay—PC12 cells were transfected as described above. A cell count of both attached and detached cells was quantified and cell viability was assessed by the trypan blue exclusion assay. Viable cells excluding the dye (unstained) and cells incorporating the dye (stained) are non-viable. Cell viability after treatment was determined by counting the total number of cells and the number of unstained, and stained cells and data are expressed as a percent of control.

Nuclear and Cytoplasmic Extracts—PC12 cells were plated in 10 ml of complete media in 100-mm tissue culture plates at 1 × 107 cells/plate. After CD treatment, nuclear and cytoplasmic extracts were made using a procedure described previously (42).

Electrophoretic Mobility Shifts (EMSAs)—EMSAs were performed as described previously (39). Briefly, equal amounts of nuclear extract (5 μg) were incubated with a [^{32}P]dCTP-labeled probe in 1 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia Biotech, Piscataway, NJ). Complexes were separated on a 5% polyacrylamide gel in high-ionic strength Tris-glycine-EDTA buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA), dried, and autoradiographed. For supershift experiments, nuclear extracts were incubated with rabbit polyclonal antibodies against either the p50 subunit (sc-114; Santa Cruz Biotechnology, Inc.) or the p65 subunit (Rel A; 100–4165; Rockland, Gilbertsville, PA) of NF-κB. Nuclear extracts were incubated with poly(dI-dC)·poly(dI-dC) and the α-^{32}P-labeled probe and then analyzed as described above.

Statistical Analysis—Significant differences between treatment groups were assessed with analysis of variance (ANOVA) by Macintosh StatView software.

RESULTS

Cell-cycle Analysis of Choline-deficient Cells—CD has been shown previously to inhibit cell growth in PC12 cells (8, 15), however the molecular mechanisms associated with this response have not been clearly determined. To characterize the nature of the CD-induced growth arrest, we examined cell cycle distribution by flow cytometric analysis. Proliferating PC12 cells were grown in media containing 130 μM choline (CS), or in media without choline (CD). In a parallel experiment PC12

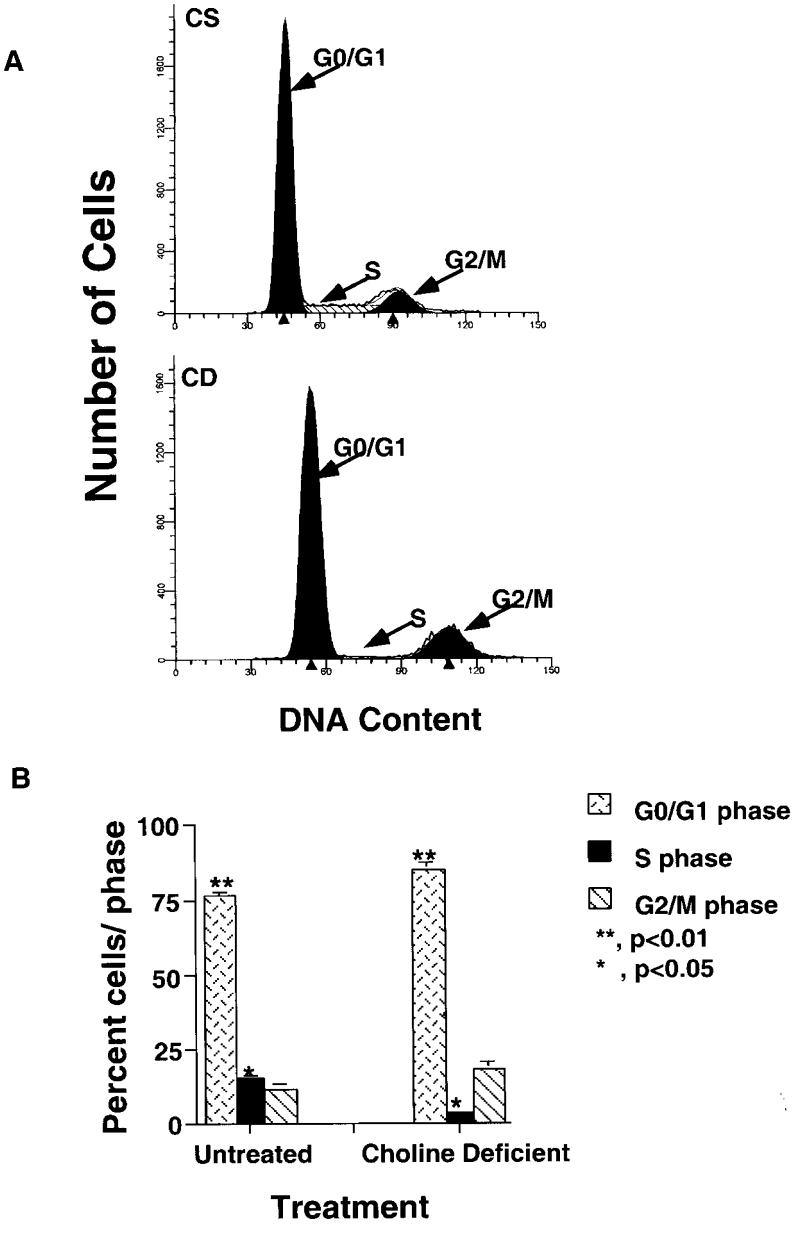

FIG. 1. **Altered cell cycle distribution after acute CD.** *A,* cells were harvested after 48 h, the DNA content was measured by propidium iodide staining (*x* axis), and the relative number of cells detected by BrdUrd incorporation (*y* axis) were analyzed. Data are representative experiments of a two-dimensional flow cytometric analysis illustrating the presence of both G_0/G_1 and G_2/M arrest in response to CD. *B,* graph represents the percentage of cells either in G_0/G_1, S, and G_2/M cell cycle phases for either choline sufficient (*CS*) or choline-deficient (*CD*) cells as represented in histogram (Fig. 1*A*). Cell cycle progression of PC-12 cells was analyzed as described under "Materials and Methods." Data are means ± S.E. from three independent studies (n = 3/group). Within cell cycle phases significance is indicated by ANOVA. *Asterisks* indicate significance at $p < 0.05$ (*) or at the $p < 0.01$ (**) level.

cells were either irradiated (5 Gy; γ-IRR) or not irradiated (Sham). As shown in the flow cytometric histogram (Fig. 1*A*), cell cycle distribution was significantly altered during CD as compared with its matched control, CS, at 48 h. In particular, during CD PC12 cells exhibited an 8-fold increase in the proportion of cells arrested in G_0/G_1 ($p < 0.0001$) and a 1.6-fold increase in G_2/M ($p < 0.0001$) (Fig. 1*B*). The number of total cells in S-phase were reduced 2.7-fold after CD ($p < 0.001$) (Fig. 1, *A* and *B*). γ-IRR is a potent inducer of a p53-mediated pathway that results in G_1 arrest, therefore it was used as a positive control (43, 44). As expected, after γ-IRR the majority of cells accumulated in both G_0/G_1 and G_2/M of the cell cycle (data not shown). These data demonstrate that CD inhibited cell cycle progression and suggest that this inhibition may occur through a p53-mediated pathway.

p53 Involvement in Choline Deficiency-induced Apoptosis and Cell Cycle Arrest—Previous evidence for the induction of apoptosis by CD was shown by growth inhibition and by the demonstration of DNA fragmentation (15, 16). Cellular growth arrest is often mediated by induction of p53, and p53-mediated arrest depends, in part, on the induction of $p21^{WAF1/CIP1}$ (45). Therefore, to study the signaling pathway involved in CD-induced cell cycle arrest and cell death, we measured the induction of p53 and $p21^{WAF1/CIP1}$ protein expression. Cytoplasmic extracts were prepared from PC12 cells that were also used for EMSAs. p53 and $p21^{WAF1/CIP1}$ protein expression in PC12 cells were examined following either CD or γ-IRR treatment by Western blot analysis. γ-IRR is known to activate a p53-mediated pathway resulting in p53 protein accumulation and was the positive control (43, 44). In PC12 cells, the expression of the p53 protein was strongly induced 24 h after CD treatment and remained elevated after 48 h (Fig. 2*A*; compare *lane 1* with *lanes 3* and *4*). As expected, p53 protein expression was dramatically increased in γ-IRR PC12 cells after 24 h (Fig. 2*A*, *lane 2*). Using the same extracts, $p21^{WAF1/CIP1}$ protein expression was induced during CD and γ-IRR in a time-dependent manner (Fig. 2*B*, *lanes 3–4*). $p21^{WAF1/CIP1}$ function has been demonstrated to be necessary for p53-mediated G_1 arrest following irradiation (45). These data are important because the induction of p53 and $p21^{WAF1/CIP1}$ protein expression in response to CD are consistent with growth inhibition previously reported

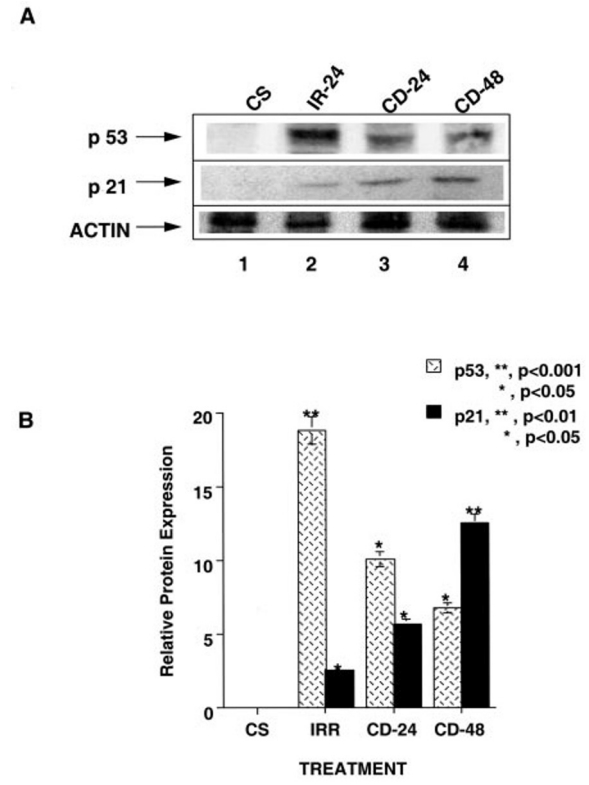

FIG. 2. **Induced p53 and p21 protein expression by CD and γ-irradiation.** *A,* Western blot analyses were performed using equal amounts of protein from PC12 cell cytoplasmic extracts using polyclonal antibodies specific for p53 and p21. The treatments and times are indicated above each lane (*CS* is choline sufficient). *Arrows* indicate detection of specific protein. Actin was used as a control for protein loading. *B,* relative p53 and p21 protein expression. Autoradiographs of p53 and p21 protein expression were scanned by densitometry and quantitated. Histogram represents protein expression after each time and treatment. Data are representative of three independent experiments. *Asterisks* indicate significance at $p < 0.001$ by ANOVA.

(15, 16) and with inhibition of cell cycle progression in the present study.

To determine whether p53 controls the observed CD-induced cell growth and apoptosis, we used a cell line that was inducible for functional p53. The H1299 human lung carcinoma cell line does not express endogenous wild type p53 (wt-p53), but has been engineered to stably express a temperature sensitive (ts) form of p53 (H1299.ts-p53). The H1299.ts-p53 cell line is well characterized and provides an effective tool for analysis of p53 function without interference from endogenous wt-p53 protein (37, 38). Expression of the mutant p53 conformation and activity occurred at 39 °C, and expression of the wild type conformation and its associated activity occurred at 32 °C (38). Controls consisted of staurosporine as the positive control and untreated media as the negative control for apoptosis (Fig. 3*A*). Evidence of DNA fragmentation was determined quantitatively in H1299.ts-p53 cells by the cell death ELISA assay. Upon the expression of wt-p53 at 32 °C, CD strongly induced apoptosis as determined by cell death ELISA (Fig. 3*B*), morphological analysis, and TUNEL assay (Fig. 3*C*). But at 39 °C, the p53 mutant significantly reduced the ability to support CD-induced apoptosis (Fig. 3, *B* and *C, upper panel*). In addition, $p21^{WAF1/CIP1}$ protein expression was induced at 32 °C during CD but not at 39 °C as indicated by Western blot analysis (data not shown). Thus, our data strongly suggest that the observed CD-induced apoptosis is a p53-mediated process in both preneuronal and epithelial cells as evidenced by the induction of both p53 and $p21^{WAF1/CIP1}$ protein expression.

Protection against Choline Deficiency-induced Cell Death by Inhibition of Cell Death—The mutant $p53^{135}$ protein can abrogate p53-mediated cell death because it functions as a dominant negative (p53DN) by inhibiting endogenous wt-p53 protein (41, 46). p53 is wild type in this PC12 cell line, therefore, it is possible that wt-p53 contributed to CD-induced cell death. To determine whether a p53-dependent mechanism was operative during CD-induced apoptosis, we examined the ability of the p53DN to rescue cells from CD-induced apoptosis. PC12 cells were transiently transfected using LipofectAMINE, and transfection efficiency, which corresponds to cell survival, was determined by using the β-gal assay. PC12 cells expressing the transiently transfected p53DN were significantly resistant to cell death, as indicated by 87% cell survival relative to the control (CS) after CD treatment as measured by the ELISA apoptosis assay (Fig. 4*A*) and by β-gal assay (Fig. 4*C*). Interestingly, these data also indicate that less than 50% of cells initially die in response to CD. Our study strongly indicates that wt-p53 was functionally inactivated by the p53DN since CD failed to induce cell death in PC12 cells, thus supporting a p53-dependent mechanism. Importantly, our data strongly suggest that endogenous p53 plays a major role in the pro-apoptotic function of acute CD.

Choline Deficiency Induced NF-κB Activity, Which Opposes CD-induced Apoptosis—In contrast to p53 activation, which is associated with the induction of apoptosis, the activation of NF-κB promotes cell survival in response to certain stimuli (29, 47). Additionally, NF-κB has been proposed to antagonize p53 responses in some (36, 48), but not all (49), experimental models. Therefore, we examined whether NF-κB activity is modulated during CD and whether this potential response can affect apoptosis during CD. The nuclear extracts used were prepared from PC12 cells previously used for Western analysis. Minimal NF-κB DNA binding was detected in untreated PC12 cells (Fig. 5*A*, *Lane 2*). As expected, TNF-α treatment alone for 30 min (Fig. 5*A*, *lane 1*) induced strong binding of the major NF-κB-specific complex identified as the p50-p65 heterodimer (data not shown). Similarly, CD treatment elicited enhanced κB-specific binding activity after 24 h (Fig. 5*A*, *lane 3*). At 48 and 72 h of CD, NF-κB DNA binding activity remained elevated above the extracts from untreated cells (Fig. 5*A*, *lanes 4* and *5*; Fig. 5*B*). In the same nuclear extracts, DNA binding of the constitutive transcription factor Oct-1 was unaffected by the presence of CD (data not shown), demonstrating that CD does not affect transcription factors in a general manner. Consistent with the DNA binding data, CD strongly induced an NF-κB-dependent luciferase reporter (3xκB-luc) in PC12 cells, as did TNF stimulation (data not shown). However, CD did not strongly induce a mutant 3xκB-luc reporter in which the three NF-κB binding sites are mutated (data not shown). Thus, the effects of CD on NF-κB DNA binding activity paralleled those observed in the NF-κB-dependent gene expression studies. These results indicate that CD activates NF-κB-dependent gene expression through the induction of NF-κB DNA binding activity.

To determine whether NF-κB was required for a cell survival response during CD, we used the super repressor form of IκBα (SR-IκBα) (29, 30) to block endogenous NF-κB activity in PC12 cells. SR IκBα inhibition of NF-κB dramatically enhanced CD-induced apoptosis, as determined by both cell death ELISA (Fig. 6*A*) and morphological analysis (Fig. 6*B*). Cell death, induced either by CD in vector controls or by SR-IκBα inhibition of NF-κB, exhibited the hallmarks of apoptosis, such as rounded cells with condensed nuclei as well as fragmented nuclei (Fig. 6*B*). These results are consistent with previous findings that inhibition of NF-κB, in association with certain

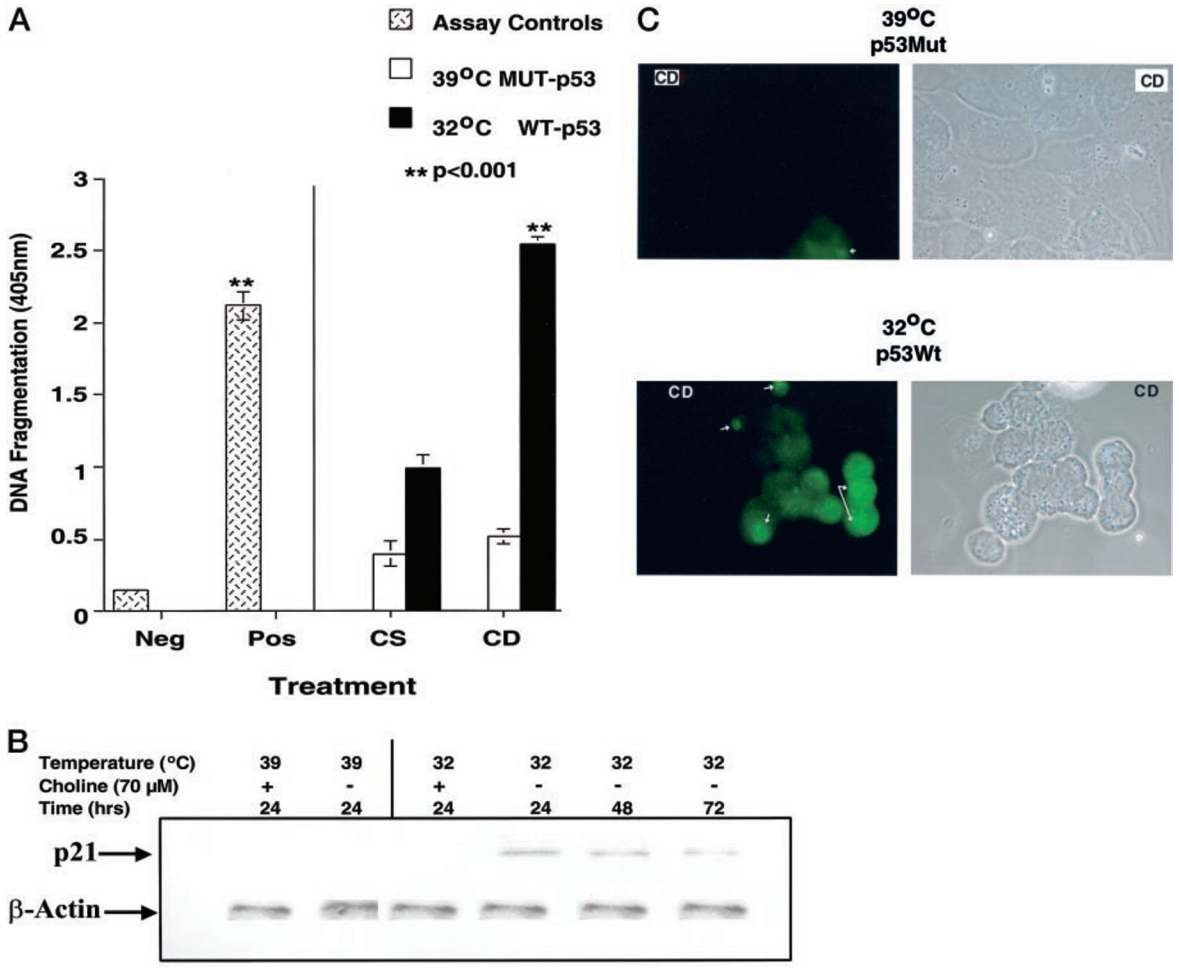

FIG. 3. **Wild type-p53 expression enhanced DNA fragmentation after CD treatment.** *A,* H1299 cells were treated with staurosporine, a protein kinase C inhibitor, as a positive control for the induction of apoptosis, and media from untreated cells was the negative control. Wild type p53 (wt-p53) was conditionally expressed in the H1299 stable cell line at 32 °C and mutant p53 (Mut-p53) expressed at 39 °C. Detection of single and double DNA strand breaks were quantitated photometrically by the ELISA *in situ* assay (Roche Molecular Biochemicals). *Asterisks* indicate significance at $p < 0.05$ (*) or at the $p < 0.01$ (**) level. Samples were run in triplicate, and data represents three independent experiments. *B,* p53-dependent CD-induced p21 expression. H1299.tsp53 cells were incubated with (+) and without (−) choline, wild type p53 (wt-p53) was conditionally expressed in the H1299 stable cell line at 32 °C, and mutant p53 (Mut-p53) was expressed at 39 °C. At indicated time points, cells were harvested, and cell lysates were prepared. Using p21 and β-actin-specific antibodies, protein expression was determined by Western blot analysis and visualized by chemiluminescence and autoradiography. β-actin was used as a control for protein loading. *C,* Wt-p53 induced apoptosis during CD. Micrograph (magnification, ×40) of stable H1299 cells expressing the temperature-sensitive p53 expression vector. Cells were cultured in the presence (+) or absence (−) of choline either at 32 °C or at 39 °C for 48 h. Apoptotic cells were detected by TUNEL (Roche Molecular Biochemicals), counterstained with fluorescein mounting medium (DAB, UNCORE), and viewed by fluorescent microscopy. Apoptotic cells are shown as round and fluorescent with condensed nuclei (*arrows*). The same field is shown by phase contrast (*right panel*).

stimuli, causes cells to undergo apoptosis (28, 39, 47). Taken together, our study may provide a mechanism whereby CD not only promote cell survival, but also subsequently promote CD-induced oncogenesis.

DISCUSSION

Little is known about nutrient-regulated apoptosis, or about the signaling mechanisms that determine whether cells arrest or undergo cell death following nutrient deficiency. Our studies lead to several important conclusions regarding the relationship among CD, p53, and $p21^{WAF1/CIP1}$ expression in growth arrest, apoptosis, and activation of the transcription factor, NF-κB. In this report, we addressed the role of p53 in mediating CD-induced growth arrest with subsequent transcription and induction of apoptosis in preneuronal and epithelial cell lines and the role of the transcription factor NF-κB.

During CD, cell cycle arrest was detected at both the G_1/S and G_2/M transitions (Fig. 1), indicating that CD is a sufficient cellular stress to negatively regulate cell cycle progression. Many forms of stress can activate the transcription factor p53 including DNA damage, activation of oncogenes, and hypoxia (50). p53 plays a significant role in transcriptional regulation of target genes that encode proteins involved in cell cycle regulation (51) and is involved in the induction of apoptosis (52). Induction of $p21^{WAF1/CIP1}$ can regulate both a G_1 arrest (53, 54) and a sustained G_2/M arrest in both a p53-dependent and p53-independent mechanism (20, 53, 55). Consistent with p53-mediated induction of $p21^{WAF1/CIP1}$, the previously reported CD-induced growth inhibition and subsequent induction of apoptosis (8, 15) support these findings of a G_1 arrest (Fig. 2). Similarly, CD also has been associated with nuclear expression of the cyclin-dependent kinase inhibitor (CdkI), $p27^{Kip1}$, in the acute CD CWSV-1 hepatocyte cell line (56). $p27^{Kip1}$ is considered an essential component of the TGF-β1 signaling pathway also implicated in cell cycle arrest and apoptosis (57). Although the apoptotic effects of CD have previously been described in an immortalized hepatocyte cell line (CWSV1) without functional p53 and in the Hep3B hepatocyte cell line that has mutated p53 (58), these studies may suggest that p53 activation is not es-

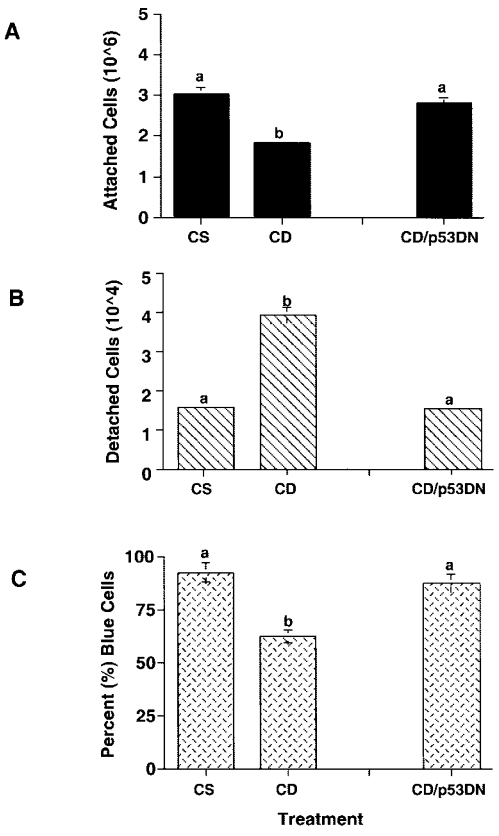

FIG. 4. **Protection from CD induces apoptosis.** PC12 cells were transfected with either the empty/control vector (*CS* and *CD*) or with the p53DN expression vector. Transfected cells were treated with (*CS*) or without choline (*CD*) for 48 h. A cell count of both attached (*Panel A*) and detached cells (*Panel B*) were quantified and cell viability was assessed by the trypan blue exclusion assay. Data represents means ± S.E. from independent experiments. A parallel study included the β-galactosidase *in situ* assay as a measure of transfection efficiency and correspondingly to cell survival (*Panel C*). Data are expressed as a percent. Different superscripts indicate significance between treatment groups at $p < 0.01$ level by ANOVA.

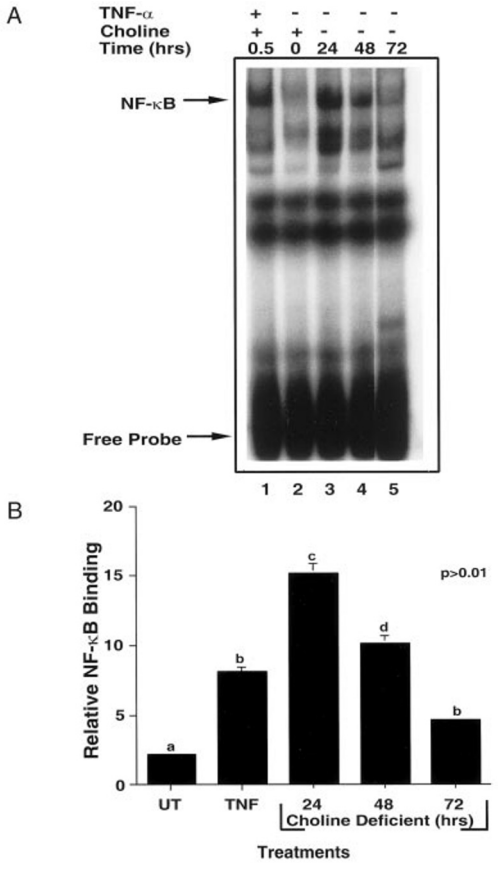

FIG. 5. **Prolonged activation of NF-κB during CD.** *A*, EMSAs of equal amounts of protein from nuclear extracts using a probe containing a consensus NF-κB binding site are shown. The times and treatments are indicated above each *lane*. *Arrows* indicate the major NF-κB specific band (p50/p65) and free probe. TNFα (10 ng/ml) is a positive control for NF-κB translocation and DNA binding. Data are representative of three independent experiments. *B,* relative NF-κB DNA binding. NF-κB DNA binding activity was quantitated by PhosphorImager analysis (Molecular Dynamics) and normalized to the DNA binding activity of untreated controls (*Lane 2*). Different superscripts between treatment groups are significant at $p < 0.01$ level by ANOVA.

sential for CD-induced apoptosis in these cell lines. However, our data strongly suggest that when p53 is activated by CD it greatly enhanced the extent of apoptosis. Although perturbations in the levels of cyclin-dependent proteins have been shown to lead to loss of G_1 control and to subsequent cancer development (54, 59), the effect of CD on the expression of cell cycle regulatory proteins and their role in CD-induced carcinogenesis have not been elucidated. Therefore, future studies will investigate the effect of CD on the function of cell cycle regulatory proteins and their role in CD-induced cell transformation.

Further exploration of the effect of CD on p53 function and its role in mediating apoptosis in epithelial cells was examined using an H1299 p53-null human lung carcinoma cell line, stably expressing the ts p53 protein (37, 38). The ts-p53 expression vector is in the wt conformation at 32 °C, but in the mutant conformation at 39 °C (38). This cell line enabled analysis of p53 function without interference from endogenous wt-p53 protein. The evidence of CD-induced apoptosis (Fig. 3, *A* and *C*) and the induction of p21 (Fig. 3*B*) in these cells confirmed the activation of wt-p53 and induction of its transcriptional function. In addition, a $p53^{135}$, which functions as a dominant negative (p53DN) (41), inhibited CD-induced cell death indicating suppression of p53-dependent gene transcription (Fig. 4, *A*–C). These study observations strongly support the hypothesis that p53 transcriptionally mediates both the cell cycle arrest and apoptosis induced by CD.

NF-κB is activated by a variety of stimuli and is responsive also to oxidative stress (24, 25, 60), and it has been shown that NF-κB can activate p53 as a target gene (61). In addition, NF-κB has a role in apoptosis whether protective (29, 30) or causative (49). In our present study, acute CD treatment induced NF-κB DNA binding and NF-κB-dependent gene transcription. We suggest that NF-κB acts as a repressor of the p53-mediated cell death process induced by CD, because loss of NF-κB function increased cell susceptibility to cell death (Fig. 6, *A* and *B*). These studies demonstrate that NF-κB is required for cell survival during CD, which is consistent with previous findings that CWSV-1 hepatocytes gradually adapt to survive low choline with subsequent resistance to CD-induced apoptosis and enhanced potential for cell transformation (11) and that after 72 h of CD ~50–60% of cells have not undergone apoptosis (8). Interestingly, in a number of systems, NF-κB has been proposed to regulate $p21^{WAF1/CIP1}$ expression and its expression to confer a cell survival function (62, 63). Hence, NF-κB activation may play an important role in regulating the adaptive response of cells to CD by enhancing their survival (29, 64) in part through the coordinated up-regulation of $p21^{WAF1/CIP1}$ (62, 63). Importantly, others have shown that p53 and NF-κB can mutually suppress each other's ability to activate transcription (65–67). These data are consistent with previous studies using knockout animals that have shown that the

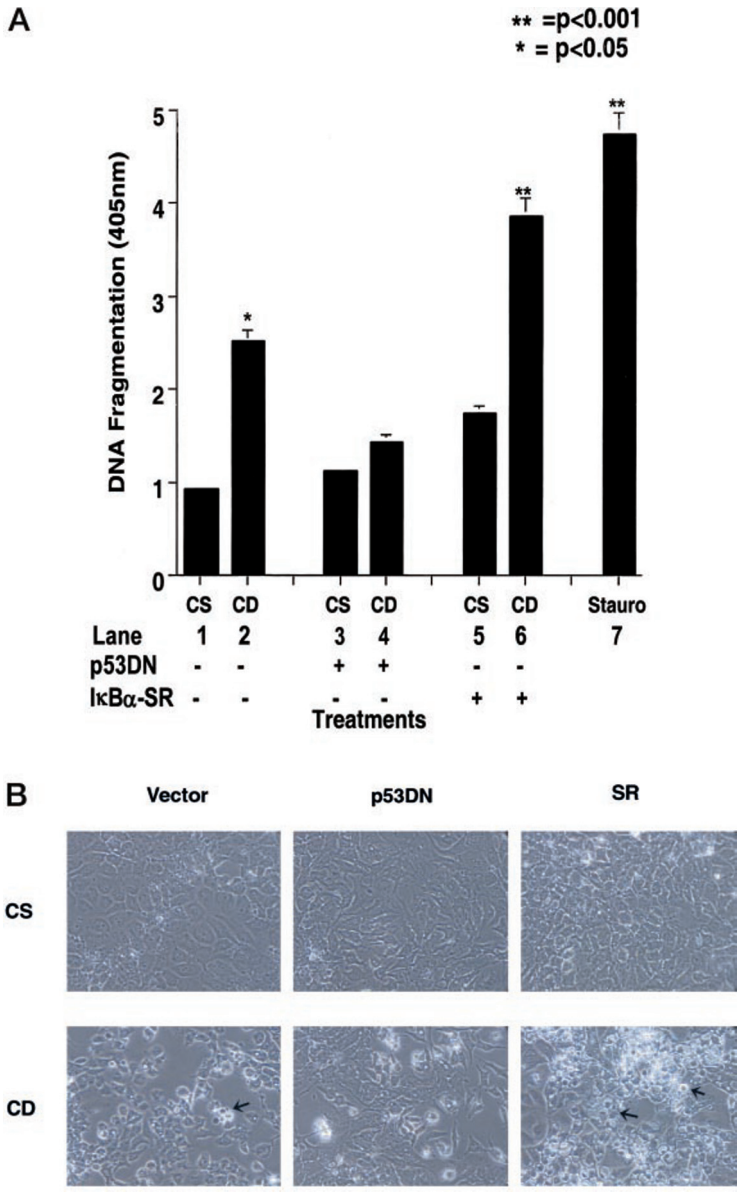

FIG. 6. **Apoptotic effects of CD in PC12 cells expressing SR-IκBα.** *A,* SR-IκBα-mediated inhibition of NF-κB enhanced DNA fragmentation. PC12 cells expressing either the p53DN or the SR-IκBα expression vectors were cultured in the presence (*CS*) or absence (*CD*) of choline for 24 h. After incubation, cell death was detected by the ELISA *in situ* apoptosis assay (Roche Molecular Biochemicals), and the percentage of apoptosis was quantitated photometrically. Staurosporine was used as a positive control for the induction of apoptosis. *Asterisks* indicate significance at $p < 0.05$ (*) or at the $p < 0.01$ (**) level. *B,* SR-IκBα inhibition of NF-κB enhances CD-induced apoptosis in PC12 cells. Micrograph panel (magnification, ×40): PC12 cells expressing either the empty vector p53DN or the SR-IκBα were treated with choline (*CS, top panel*) or without choline (*CD, lower panel*) for 24 h. Non-adherent dying cells are shown as rounding and refractive by phase contrast microscopy. Apoptotic cells are rounded with dark condensed nucleus (*arrows*).

liver undergoes massive apoptosis when the NF-κB p65 subunit is mutated (28). Our data supports the hypothesis that NF-κB is required to promote cell survival (29) and cellular transformation (30, 35), perhaps through the induction of NF-κB-regulated cell survival factors (64, 68) as well as through the regulation of $p21^{WAF1/CIP1}$, since this protein was found to be regulated in an NF-κB-dependent manner and to confer survival features in certain systems (62, 63). Thus, the enhanced NF-κB activation observed in our study may be a mechanism that not only promotes cell survival, but also may subsequently promote CD-induced oncogenesis. Whether NF-κB is persistently activated in cells undergoing nutrient deficiency-induced cell transformation is unknown and will be addressed in future studies. Because many nutrient deficiencies are associated with the development of disease, perhaps the induction of NF-κB may be a molecular mechanism whereby dietary deficiency promotes disease progression through altered gene expression.

Our results establish a molecular connection between the two well known transcription factors, p53 and NF-κB, through acute CD. The present studies provide evidence that CD-induced apoptosis, both in preneuronal and in epithelial cells, is under opposing regulation by p53 and NF-κB. This opposing regulation during CD supports the finding of antagonism between NF-κB and p53 (48). The importance of this finding is that a sub-population of cells are induced to undergo p53-dependent cell death, but in the surviving cells the activation of NF-κB suppresses the p53 effect as an anti-apoptotic factor. Consistent with this finding are several reports that indicate that activation of NF-κB protects cells against programmed cell death induced by cytokines (29), ionizing radiation (69), or cytotoxic drugs in some cancer cell lines (31). The mechanisms that drive these CD-treated cells to resist apoptosis and therefore survive with subsequent progression of initiated cells to form carcinomas have been poorly understood. These studies provide a molecular mechanism by which CD may induce a cell death response as well as a cell survival response and demonstrate a potential process whereby CD may lead to oncogenesis and/or inflammation.

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