The carcinogenicity of nickel compounds has been well documented both in vitro and in vivo; however, the molecular mechanisms by which nickel compounds cause cancers are far from understood. Because suppression of apoptosis is thought to contribute to carcinogenesis, we investigated the mechanisms implicated in nickel-induced anti-apoptotic effect in human bronchial epithelial (Beas-2B) cells. We found that exposure of Beas-2B cells to nickel compounds resulted in increased cyclooxygenase-2 (COX-2) expression and that small interfering RNA (siCOX-2) knockdown of COX-2 expression resulted in increased cell sensitivity to nickel-triggered cell apoptosis, demonstrating that COX-2 induction has an anti-apoptotic effect on Beas-2B cells. Overexpression of IKKβ-KM, a kinase inactive mutant of IKKβ, blocked NF-κB activation and COX-2 induction by nickel compounds, indicating that activated NF-κB may be a mediator for COX-2 induction. To further explore the contribution of the NF-κB pathway in COX-2 induction and in protection from nickel exposure, mouse embryonic fibroblasts deficient in IKKα, IKKα, p65, and p50 were analyzed. Loss of IKKβ impaired COX-2 induction by nickel exposure, whereas knockout of IKKα had a marginal effect. Moreover, the NF-κB pathway was critical for nickel-induced COX-2 expression. In addition, a deficiency of IKKβ or p65 rendered cells more sensitive to nickel-induced apoptosis as compared with those in wild type cells. Finally, it was shown that reactive oxygen species H₂O₂ were involved in both NF-κB activation and COX-2 expression. Collectively, our results demonstrate that COX-2 induction by nickel compounds occurs via an IKKβ/p65 NF-κB-dependent but IKKα- and p50-independent pathway and plays a crucial role in antagonizing nickel-induced cell apoptosis in Beas-2B cells.

Aerosols of nickel salts can be generated in electroplating and electrolysis areas of nickel refineries (1). The release of nickel into the environment represents a potential for nonoccupational exposure (2). The routes for nickel uptake include inhalation, ingestion, and dermal penetration (1, 3). Epidemiological studies have associated occupational exposure to nickel compounds to elevated incidences of human cancer such as lung and nasal cancers (4). Numerous studies from cell culture models and experimental animal models have also confirmed the carcinogenic potency of nickel compounds (4, 5). Although the mechanisms implicated in the carcinogenic effect of nickel compounds are not well understood, it is accepted that the carcinogenic effects of nickel occur through alterations in cancer development-related gene expression (6). On the other hand, it has been reported that nickel compounds can promote the generation of reactive oxygen species (ROS), which can regulate the expression of specific genes related to tumor development (7).

Apoptosis plays an essential role as a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells (8, 9). Cyclooxygenase-2 (COX-2) is implicated in the suppression of apoptosis, in some experimental systems, leading to the development of cancer (10, 11). Previous studies have documented that COX-2 is constitutively overexpressed in a variety of human malignancies, especially in primary lung carcinoma (12–14). In the current study we utilized human bronchial epithelial Beas-2B cells to define whether nickel compounds are able to promote survival by inducing COX-2 expression and to define the signals regulating nickel-induced COX-2 expression.

**MATERIALS AND METHODS**

**Plasmids, Antibodies, and Other Reagents**—HA-tagged full-length IKKβ (HA-IKKβ) were provided by Dr. Zheng-Gang Liu (NCI/National Institutes of Health, Bethesda, MD) (15). Mitoxantrone was purchased from Bachem and fetal bovine serum (FBS) was purchased from Hyclone. The abbreviations used are: ROS, reactive oxygen species; COX-2, cyclooxygenase-2; AP, activator protein; siRNA, small interfering RNA; IKK, IkB kinase; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; HA, hemagglutinin; RT, reverse transcription; WT, wild type; MEF, mouse embryonic fibroblast; MAPK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase; mCat, mitochondrial catalase.
chondrial catalase expression vector pZeoSV/mCAT was described previously by Rodríguez et al. (16). The antibodies against phospho-IKKα/β, IKKα, IKKβ, phospho-1κBα, 1κBα, caspase-3, and PARP were purchased from Cell Signaling Technology (Beverly, MA). Anti-HA antibody was purchased from Upstate Biotechnology (Lake Placid, NY); anti-FLAG and anti-β-actin antibodies were obtained from Sigma; anti-COX-2 antibody was purchased from Cayman Chemical (Ann Arbor, MI). Anti-catalase antibody was purchased from Calbiochem (EMD Biosciences, Inc., La Jolla, CA). Nickel compounds were purchased from Aldrich; and substrate for the luciferase assay was purchased from Promega (Madison, WI).

Cell Culture and Transfection—Beas-2B cells, wild type (WT) mouse embryonic fibroblasts (MEFs), and their stable transfectants were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Calbiochem) supplemented with 10% fetal bovine serum (FBS), 5% penicillin/streptomycin, and 2 mM L-glutamine (Invitrogen). All MEFs and their transfectants were maintained in H-DMEM with 5% CO₂. To block IKK/NF-κB pathway activation, IKKβ-KM expressing plasmid was used to transfect Beas2B cells and WT cells together with NF-κB gene reporter plasmid or COX-2 gene reporter plasmid. Stable transfection was performed with Lipofectamine reagent according to the manufacturer’s instruction. After co-transfection with hygromycin B-resistant plasmid, cells were subjected to hygromycin B drug selection to generate stable transfectants. The stable transfectants were identified by analyzing basal luciferase activity or selection to generate stable transfectants. The stable transfectants were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Calbiochem) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine.

Gene Reporter Assays—Confluent monolayers of stable luciferase reporter transfectants were trypsinized, and 8 × 10⁴ viable cells suspended in 100 μl of 10% FBS/DMEM were added to each well of 96-well plates. Plates were incubated at 37 °C in a humidified atmosphere with 5% CO₂. To block IKK/NF-κB pathway activation, IKKβ-KM expressing plasmid was used to transfect Beas2B cells and WT cells together with NF-κB gene reporter plasmid or COX-2 gene reporter plasmid. Stable transfection was performed with Lipofectamine reagent according to the manufacturer’s instruction. After co-transfection with hygromycin B-resistant plasmid, cells were subjected to hygromycin B drug selection to generate stable transfectants. The stable transfectants were identified by analyzing basal luciferase activity or selection to generate stable transfectants. The stable transfectants were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Calbiochem) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine.

Western Blot Assay—2 × 10⁵ cells were cultured in each well of 6-well plates to 70–80% confluence. The culture medium was replaced with 0.1% FBS/DMEM. After being cultured for 24 h, the cells were exposed to nickel compounds. The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with a rabbit-specific antibody against target protein. The protein band, specifically bound to the primary antibody, was detected using an anti-rabbit IgG-AP-linked antibody and an ECF Western blotting system (Amersham Biosciences).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted with Trizol reagent (Invitrogen), and cDNAs were synthesized with ThermoScript™ RT-PCR system (Invitrogen). For detection of COX-2 expression, a pair of oligonucleotides (5’-tgaacccctctcacaca-3’ and 5’-aactgtagggtaggtgctg-3’) were designed according to human COX-2 gene sequence. The human β-actin cDNA was amplified at the same time by the primers 5’-gaggaatggccagctat-3’ and 5’-gctcaggaggaatgtct-3’. Cell Death Assay—Beas-2B cells treated with nickel compounds were collected by pooling the cells from the culture medium as well as the trypsORIZED adherent cells. Dead cells were counted by the trypan blue exclusion method and flow cytometric analysis following propidium iodide staining of the nuclei. Briefly, the cells were fixed in ice-cold 80% ethanol at −20 °C overnight. The fixed cells were permeabilized in buffer containing 100 mM sodium citrate/0.1% Triton X-100 at room temperature for 15 min as well as RNase A (0.2 mg/ml) (Sigma) for 10 min, stained with propidium iodide (50 μg/ml) at 4 °C for at least 1 h, and then analyzed using the Epics XL FACS (Beckman-Coulter, Miami, FL) as described in our previous publication (3, 17).

H₂O₂ Staining Assay—Beas-2B transfectants were seeded (2 × 10⁴) into each well of a 96-well plate. After cell density reached 80–90%, the cells were washed thoroughly with PBS and incubated with dichlorofluorescein diacetate (DCFH-DA) at 10 μM in PBS (stock concentration is 10 mM in Me₂SO) for 20 min. Cells were then washed with PBS to remove the dye completely and exposed to UVC radiation (60 J/m²). The cells were incubated at 37 °C for another 10 min, and the oxidative product was detected by using an HTS7000 Bio-Assay reader (PerkinElmer Life Sciences) with excitation 488 nm and emission 530 nm. The results were expressed as relative H₂O₂ production compared with the cells without exposure to UVC radiation.

Electrophoretic Mobility Shift Assays—Nuclear proteins were prepared with a Cellytic™ NuCLEAR™ extraction kit (Sigma) following the manufacturer’s protocols. 5 μg of nuclear protein was subjected to a gel shift assay by incubating it with 1 μg of poly(dI-dC) DNA carrier in DNA-binding buffer (10 mM Tris, pH 8.0, 150 mM KCl, 2 mM EDTA, 10 mM MgCl₂, 10 mM dithiothreitol, 0.1% bovine serum albumin, 20% glycerol) in a final volume of 10 μl on ice for 10 min. Then, 10⁶ cpm (10⁶ cpm/μg) of the ³²P-labeled double-stranded oligonucleotide (2 μl) was added, and the reaction was incubated at room temperature for 30 min. For competition experiments, a 20-fold molar excess amount of the unlabeled oligonucleotide was added before the addition of the probe. DNA-protein complexes were resolved by electrophoresis in 5% nondenaturing glycerol/polyacrylamide gels. The synthetic oligonucleotides (5’ to 3’) used as probe binding to NF-κB was GAGTGAGGCGACTTCCAGGC.
FIGURE 1. Nickel exposure rendered Beas-2B cells resistant to the pro-apoptotic effects by induction of Cox-2. 8 × 10⁵ Beas-2B-COX-2 mass1 cells were seeded into each well of a 96-well plate. After being cultured at 37 °C overnight, the cells were treated with either 0.5 mM NiCl₂ or 2 µg/cm² NiS for various time points as indicated (a). For dose-response studies, the cells were treated with various doses of nickel compounds for 48 h (b). The cells were then extracted with lysis buffer, and luciferase activity was measured using the Promega luciferase assay kit as described under “Materials and Methods.” The results were expressed as the induction of COX-2 relative to the medium control (relative COX-2 induction). Each bar indicates the mean ± S.D. of triplicate wells. The asterisk indicates a significant increase from medium control cells (*p < 0.05). Beas-2B cells and their transfectants (2 × 10⁵) were seeded into each well of 6-well plates and cultured in 10% FBS/DMEM at 37 °C. When the cell density reached 70 – 80%, the culture medium was replaced with 0.1% FBS/DMEM. After being cultured overnight, the cells were exposed to nickel compounds at the indicated dosages for 24 or 48 h. The total RNA was extracted for RT-PCR (c), or the cells were extracted for preparation of Western blot (d, e, and h). The protein band, specifically bound with the primary antibodies, was detected by using anti-rabbit IgG-AP-linked secondary antibody and an ECF Western blotting system, or the cell morphological changes were observed, and photos were taken under the microscope (f). Cell apoptosis was determined by flow cytometric analysis (g).
RESULTS

Nickel Exposure Rendered Beas-2B Cells Resistant to the Pro-apoptotic Effects by Induction of COX-2—Previous studies have demonstrated that environmental and occupational exposure to nickel compounds is associated with an increased risk of lung cancer (3). Epidemiological evidence also indicates that COX-2 is constitutively overexpressed in most human lung cancers (18–20). Thus, it was of interest to investigate the potential contributions of elevated COX-2 protein in response to nickel-caused biological effects and their mechanisms. We first sought to determine whether nickel compounds induce COX-2 expression in human bronchial epithelial cells. As shown in Fig. 1, a and b, treatment of Beas-2B cells with water-soluble NiCl₂ or water-insoluble NiS led to marked increases of COX-2 transcription as monitored using a COX-2-luciferase reporter. The nickel-dependent induction of COX-2 was both dose- and time-dependent. RT-PCR and Western blotting confirmed that endogenous COX-2 levels are also nickel-responsive (Fig. 1, c and d). These results verified that nickel exposure led to an increase in the COX-2 mRNA and protein expression in Beas-2B cells.

To evaluate the role of nickel-dependent COX-2 production in cell survival, specific human COX-2 siRNA (siCOX-2) was used to decrease COX-2 level. Stable transfection of siCOX-2 led to an almost complete block of COX-2 expression in response to nickel exposure, whereas control siRNA had no effect (Fig. 1 e). Knockdown of COX-2 protein expression by siCOX-2 resulted in significant increases in the sensitivity of Beas-2B cells to nickel-triggered cell death (Fig. 1 f). Analysis of apoptosis using either propidium iodide staining (to evaluate hypodiploid DNA content) or caspase-3 or PARP cleavage demonstrated loss of COX-2 nickel-induced cell death (Fig. 1, g and h). These results show that COX-2 is responsible for preventing cell death in the presence of nickel.

Activation of IKK/NF-κB Pathway Is Required for COX-2 Induction by Nickel Compounds in Beas-2B Cells—The COX-2 promoter region contains several NF-κB binding sites thought to be involved in the regulation of its expression (21, 22). Thus, we next evaluated the role of NF-κB in nickel-induced COX-2 expression in Beas-2B cells. NF-κB is normally sequestered in the cytoplasm as an inac-
Mechanism and Role of COX-2 Induction in Nickel Response

**FIGURE 3. Essential role of IKKβ/NF-κB pathway in COX-2 induction by nickel compounds in Beas-2B cells.** IKKβ-KM transfectants were identified as compared with Beas-2B/vector control cells using Western blot with FLAG-specific antibodies as indicated (a). For determination of IKKβ-KM inhibitory effect on IκBα phosphorylation and degradation, 2 × 10^6 Beas-2B/NF-κB mass1 cells and Beas-2B/NF-κB-IKKβ-KM mass1 were seeded into each well of 6-well plates and cultured in 10% FBS/DMEM at 37 °C. When cell density reached 70–80%, the culture medium was replaced with 0.1% FBS/DMEM. After being cultured for 48 h, the cells were exposed to either 0.5 mM NiCl2 or 2 μg/cm² NIS (c). The cells were extracted, and Western blot assay was performed as described in the legend to Fig. 1. For determination of NF-κB activity, 8 × 10^5 Beas-2B/NF-κB mass1 and Beas-2B/NF-κB-IKKβ-KM mass1 cells were seeded into each well of a 96-well plate. After being cultured at 37 °C overnight, the cells were treated with nickel compounds for 24 h as indicated (d and e). Luciferase activity was measured as described under “Materials and Methods.” For detection of COX-2 protein expression (f), 2 × 10^5 Beas-2B/NF-κB mass1 and Beas-2B/NF-κB-IKKβ-KM mass1 cells were seeded into each well of a 6-well plate and cultured at 37 °C until 70–80% confluence. The culture medium was replaced with 0.1% FBS/DMEM and cultured for 24 h. The cells were exposed to nickel compounds for 48 h, washed once with ice-cold PBS, and then extracted with sample buffer. Western blot was carried out as described under “Materials and Methods.”

NF-κB activation requires the phosphorylation IκBα by IκB kinase (IKK) leading to IκBα degradation and subsequent release and nuclear translocation of NF-κB, where it regulates the transcription of its target genes (23, 24). Our findings indicate that exposure to nickel leads to an increase in IKK phosphorylation, IκBα phosphorylation, and degradation (Fig. 2, a and b). In turn, this results in NF-κB transactivation (Fig. 2, c and d) in Beas-2B cells, consistent with our previous finding that nickel exposure may lead to NF-κB activation (25). Furthermore, analysis of NF-κB binding activity using an electrophoretic mobility shift assay indicated (Fig. 2e) that NiCl2 treatment enhanced binding in a dose-dependent manner that was abolished in the presence of a 20-fold excess of unlabeled probe. The specificity of this binding was further confirmed in the supergel shift assay with antibody specific for the p65 subunit of NF-κB (Fig. 2e).

NF-κB activation in the time course studies reached a peak at 24 h after exposure to nickel, which occurred earlier than the COX-2 induction, suggesting that NF-κB may play a role in COX-2 induction in response to nickel. To determine the role of IKK/NF-κB pathway in COX-2 induction, an inactive mutant of IKKβ, IKKβ-KM, was utilized to establish stable Beas-2B transfectants. Fig. 3a confirmed the overexpression IKKβ-KM using FLAG-tagged analysis. Overexpression of IKKβ-KM in Beas-2B cells significantly blocked IkBα phosphorylation and degradation in response to nickel compounds (Fig. 3b and c). Furthermore, overexpression of IKKβ-KM impaired NF-κB transactivation (Fig. 3d and e). Consistent with the blockage of IKK/NF-κB pathway activation, nickel-induced COX-2 expression was also blocked (Fig. 3f). Collectively, these data indicate that the IKK/NF-κB pathway plays an important role in COX-2 induction by nickel compounds.

IKKβ, but Not IKKα, Plays a Major Role in COX-2 Induction by Nickel Compounds—To provide direct evidence for the requirement for the IKK/NF-κB pathway in nickel-induced COX-2 expression, we used several IKK/NF-κB gene knock-
out cell lines (MEFs). Similar to the results obtained with Beas-2B cells, COX-2 transcriptional induction by nickel exposure was significantly increased in a time- and dose-dependent manner in the WT MEFs (Fig. 4, a–c). Activation of the IKK/NF-κB pathway in WT MEFs was also demonstrated (Fig. 4, d–g). Furthermore, NF-κB activation preceded COX-2 induction. Overexpression of IKKβ-KM in WT MEFs (Fig. 4h) not only impaired IκBα phosphorylation and degradation (Fig. 4i) but also blocked COX-2 protein expression induced by nickel compounds (Fig. 4j). The IKK complex consists of two highly homologous kinase subunits, IKKα and IKKβ, and a nonenzymatic regulatory component, IKKγ/NEMO (26). It has been established that two NF-κB activation pathways exist (27). The first, the classical pathway, is normally triggered in response to microbial and viral infections or by exposure to proinflammatory cytokines that activate the tripartite IKK complex, leading to phosphorylation-mediated IκBα degradation. This pathway, which mostly targets p50-RelA and p50-c-Rel dimers, depends mainly on IKKβ activity (28). The second alternative path-

FIGURE 4. IKKβ/NF-κB pathway mediated COX-2 induction by nickel compounds in WT MEFs. For COX-2 luciferase reporter assay (a and b), 8 × 10^3 WT/COX-2 mass1 cells were seeded into each well of a 96-well plate. After being cultured at 37 °C overnight, the cells were treated with either NiCl_2 or NIS. The cells were then extracted with lysis buffer, and luciferase activity was measured using the Promega luciferase assay as described in the legend for Fig. 1. For determination of COX-2 protein expression (c) and IκBα/β phosphorylation (d and e), WT cells (2 × 10^5) were seeded into each well of 6-well plates and cultured in 10% FBS/DMEM at 37 °C. When cell density reached 70–80%, the culture medium was replaced with 0.1% FBS/DMEM. After being cultured for 24 h, the cells were exposed to various concentrations of nickel compounds for indicated time periods. Western blot was performed as described in the legend to Fig. 1. For the assay of NF-κB activity in WT cells (f and g), WT-NF-κB mass1 cells (8 × 10^3) were seeded into each well of 96-well plates and cultured in 10% FBS/DMEM at 37 °C for 24 h. Cells were treated with nickel compounds. The cells were extracted with lysis buffer, and luciferase activity was measured. h, IKKβ-KM transfectants were identified using Western blot as described for Fig. 3a. 2 × 10^5 WT and WT-IKKβ-KM mass1 cells were seeded into each well of a 6-well plate and cultured at 37 °C until 70–80% confluence. The cell culture medium was replaced with 0.1% FBS/DMEM and either cultured for 48 h before exposure to nickel compounds for 60 min (i) or cultured for 24 h followed by nickel compounds exposure for 48 h (j). The cells were washed once with ice-cold PBS, extracted with sample buffer, and then subjected to Western blot analysis.
Mechanism and Role of COX-2 Induction in Nickel Response

way leads to selective activation of p52-RelB dimers by inducing the processing of the NF-κB2/p100 precursor protein, which heterodimerizes with Rel B in the cytoplasm (29). This pathway is triggered by certain members of the tumor necrosis factor (TNF) cytokine family through selective activation of IKKβ homodimers by the upstream kinase NIK (30). We next addressed the role of these two pathways in NF-κB-mediated COX-2 induction by nickel compounds by transfecting WT, IKKα/−/−, and IKKβ/−/− MEFs. As shown in Fig. 5, a–e, compared with WT MEFs, both NF-κB activity and COX-2 transcription was dramatically impaired in IKKβ/−/− MEFs in response to nickel compounds. However, only a marginally inhibitory effect was observed in IKKα/−/− MEFs, suggesting that IKKβ is a major mediator for COX-2 induction by nickel compounds. To further confirm this finding, IKKβ expression plasmid was used to transfect IKKβ/−/− MEFs (Fig. 5f). As shown in Fig. 5g, reconstituted expression of IKKβ in IKKβ/−/− MEFs restored the COX-2 induction by nickel compounds, demonstrating that IKKβ is required for nickel-induced COX-2 expression. These findings indicate that nickel-induced NF-κB activation is via the classical IKKβ/NF-κB pathway, which, in turn, mediates COX-2 expression.

p65NF-κB, but Not p50NF-κB, Is Critical for COX-2 Induction by Nickel Compounds—NF-κB is a homo- or heterodimer formed from a multigene family that encodes five structurally related proteins: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel (Rel), and RelB. p50 and p65 are the two predominant NF-κB components expressed in a variety of cell types (23, 24). It has been documented that p50 and p65 have different roles in regulating NF-κB transcriptional activities and mediates different biological effects of the IKKβ/NF-κB signaling pathway under certain stimulatory conditions (31, 32). To further clarify the roles of p50 and p65 in nickel-induced COX-2 expression, MEFs from p50 and p65 gene knock-out mice were utilized.
Mechanism and Role of COX-2 Induction in Nickel Response

Although epidemiological studies have demonstrated that exposure to nickel is associated with increased risk of human lung cancer, the mechanism involved in the carcinogenic effects of nickel compounds remains obscure. In the present study, we found that COX-2 protein expression was significantly induced in both human Beas-2B cells and MEFs upon nickel treatment. Knockdown of COX-2 demonstrated that COX-2 was responsible for rendering cells resistant to nickel-dependent apoptosis. We also found that the IKKβ/p65NF-κB-dependent pathway was responsible for COX-2 induction and that H2O2 activated this pathway. Consistent with their roles in the induction of COX-2 expression, the ROS/IKKβ/p65NF-κB pathway was also critical for antagonizing nickel-triggered cell apoptosis.

Several studies indicate that apoptosis may represent a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells that have been improperly induced to divide by factors such as carcinogens (8, 9). The apoptotic disruption may allow damaged cells to escape inappropriately from apoptosis and potentially to proliferate, further providing initiating events in carcinogenic development (34). Thus, it may lead to an accumulation of genetically damaged cells that have a potential to become malignant (35). Many agents, such as phenobarbital, peroxisome proliferators, cyproterone acetate, and dichloroacetic acid, suppress the basal rate of apoptosis in normal lung tissue (18–20). The contribution of COX-2 to these processes may be because of COX-2-mediated production of prostaglandins, with subsequent conversion of pro-inflammatory eicosanoids to increase tumor cell invasiveness (28). In the current study, we found that both soluble NiCl2 and insoluble NiS were able to increase COX-2 expression. We also demonstrated that COX-2 induction by nickel compounds contributes to the protection of cells from apoptosis, as evidenced by the findings that knockdown of COX-2...
expression by its siRNA leads to an increased sensitivity of Beas-2B cells to apoptosis triggered by nickel compounds. This notion was further supported by the finding that blockage of the IKKβ/H9252/p65 pathway, either by overexpression of IKKβ-KM or by knock-out of IKKβ or p65, led to an impairment of nickel-induced COX-2 expression and a marked increase in nickel-mediated apoptosis. Considering the importance of apoptosis in suppression of cancer develop-

FIGURE 7. IKKβ and p65, but not IKKα and p50, were mediators of the anti-apoptotic effect upon nickel exposure. $2 \times 10^5$ IKKα−/−, IKKβ−/−, p50−/−, p65−/−, and their WT control cells were seeded into each well of 6-well plates and cultured in 10% FBS/DMEM at 37 °C. After cell density reached 70–80%, the culture medium was replaced with 0.1% FBS/DMEM. After being cultured for 24 h, cells were exposed to nickel compounds for 48 h. The cell morphological changes were observed, and photos were taken (a and d). The percentage of cell death was counted and calculated with trypan blue staining analysis (b and e). Cleavage of caspase-3 and PARP were detected by Western blot assay as described under “Materials and Methods” (c and f).
Mechanism and Role of COX-2 Induction in Nickel Response

To summarize, we have demonstrated here that exposure to nickel compounds may, at least partially, contribute to nickel-induced human lung carcinogenesis by rendering the nickel-treated cells resistant to apoptosis.

Because the COX-2 promoter region contains a canonical TATA-box and multiple regulatory elements, which can be recognized by transcription factors such as AP-1, NF-κB, NF-IL-6/C/EBP (nuclear factor interleukin-6/CCAAT/enhancer-binding protein), and CREB (cAMP-response element-binding protein) (46–48). The results of the gene reporter assay showed that nickel exposure is not able to induce AP-1 until 72 h, whereas COX-2 expression reaches the maximum induction at 24–48 h upon nickel exposure (25), indicating that AP-1 activation occurs in the late phase of nickel exposure as compared with the time points for COX-2 induction in Beas-2B cells. This notion has been supported by the report that nickel exposure is not able to activate MAPKs, including ERK (extracellular signal-regulated kinase), JNK (c-Jun NH$_2$-terminal kinase), and p38, as well as their downstream transcription factor c-Jun and ATF-2 in BEAS-2B cells (49). Taking these findings together, we can rule out the potential involvement of the MAPK/AP-1 pathway in nickel-induced COX-2 expression. In contrast NF-κB activation was significantly induced in cells treated with nickel compounds and reached its peak prior to that of COX-2 induction, revealing that NF-κB might be involved in COX-2 induction in the cell response to nickel compounds. We also demonstrated that the IKKβ/NF-κB pathway was responsible for nickel-induced COX-2 expression, because blockage of IKK activation by overexpression of IKKβ-KM resulted in a dramatic impairment of NF-κB activation and COX-2 induction either in Beas-2B cells or WT MEFs. Moreover, we found that IKKβ, but not IKKα, was a major mediator of nickel-induced COX-2 expression, using IKKβ- and IKKα-deficient MEFs. It has been established that the classical pathway of NF-κB activation depends on the function of the homodimer of IKKβ or heterodimer of IKKβ and IKKα, whereas an alternative pathway depends on the homodimer of IKKα. Thus, we concluded that induction of COX-2 by nickel compounds was mediated by the IKKβ/NF-κB classical pathway.

The NF-κB family is composed of five structurally related members, p50, p52, p65, c-Rel, and Rel-B. Among these, p65 and p50 are the two major NF-κB components that have different roles in regulating the NF-κB transcriptional activities and that mediate different biological effects of the IKKβ/NF-κB signaling pathway under conditions of infection, inflammation, and stress (50–53). In the current study, we found that p65 was required for COX-2 induction by nickel compounds, whereas p50 was not involved in this biological effect. Thus, our results demonstrate that COX-2 induction by nickel compounds is specifically mediated by the IKKβ/p65-dependent pathway.

ROS is one of the important determinants in the regulation of cell signaling pathways involved in proliferation, apoptosis, transformation, and senescence (54). ROS includes superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH$^-$) (54, 55). Intracellular levels of ROS are influenced by a number of endogenous and exogenous processes and are regulated by several radical scavenging enzymes (54). Exogenous agents that induce ROS generation include chemical and physical carcinogens and various cytokines. Von Knethen et al. (56) reported that intracellular superoxide produced by macrophage exposed to S-nitrosoglutathione promotes COX-2 expression by the activation of AP-1 and NF-κB. The involvement of intracellular ROS in nickel carcinogenesis has been well reviewed by various groups (57–59). Our current studies indicate that ROS mediate NF-κB activation and COX-2 expression triggered by nickel compounds. To our knowledge, this is the first report to demonstrate nickel induction of COX-2 expression via the ROS/NF-κB pathway, which may contribute to the carcinogenic activity of nickel compounds in the lung.

To summarize, we have demonstrated here that exposure to nickel is able to induce COX-2 expression through the ROS/IKKβ/p65-dependent pathway. Because COX-2 induction mediates an anti-apoptosis effect, we anticipate that it may con-
Mechanism and Role of COX-2 Induction in Nickel Response

...tribute to the carcinogenic effect of nickel compounds. These findings will not only deepen our understanding of the mechanisms implicated in nickel-induced carcinogenesis in human lung tissue but will also help in determining whether we can use NF-κB or COX-2 as targets for chemoprevention and therapy of nickel-induced human lung cancer.

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