Acquired Activation of the Akt/Cyclooxygenase-2/Mcl-1 Pathway Renders Lung Cancer Cells Resistant to Apoptosis

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ABSTRACT

Acquired apoptosis resistance plays an important role in acquired chemoresistance in cancer cells during chemotherapy. Our previous observations demonstrated that acquired tumor necrosis factor-related apoptosis-inducing ligand resistance in lung cancer cells was associated with Akt-mediated stabilization of cellular FLICE-like inhibitory protein (c-FLIP) and Mcl-1. In this report, we determined that these cells also have acquired resistance to apoptosis induced by chemotherapeutics such as cisplatin and doxorubicin (Adriamycin), which was detected in vitro in cell cultures and in vivo in xenografted tumors. We further found that cyclooxygenase-2 (COX-2) is dramatically overexpressed in cells with acquired apoptosis resistance. COX-2 seems to be a crucial mediator in acquired apoptosis resistance because suppressing COX-2 activity with a chemical inhibitor or reducing COX-2 protein expression level with COX-2 small interfering RNA dramatically alleviated resistance to therapeutic-induced apoptosis. Inhibiting Akt markedly suppressed COX-2 expression, suggesting COX-2 is a downstream effector of this cell survival kinase-mediated apoptosis resistance. Furthermore, the expression of Mcl-1 but not c-FLIP was significantly reduced when COX-2 was suppressed, and knockdown of Mcl-1 substantially sensitized the cells to apoptosis. Our results establish a novel pathway that consists of Akt, COX-2, and Mcl-1 for acquired apoptosis resistance, which could be a molecular target for circumventing acquired chemoresistance in lung cancer.

Apoptosis is an evolutionarily conserved cell suicide procedure that multicellular animals use to eliminate damaged, infected, and unwanted cells. Because it is the most effective way in limiting the expansion of genome-damaged or gene-mutated cells, it is believed that apoptosis plays a critical role in deterring cancer development (Fulda, 2009). However, cancer cells readily escape the body's natural defense mechanism. Cancer cells gain apoptosis resistance (primary) through dysfunctional apoptosis pathways and/or by elevating survival signals stemming from the acquisition of genetic and epigenetic aberrations acquired during transformation (Fulda, 2009). In addition, cancer cells acquire apoptosis resistance during chemotherapy, the mechanism of which is not well understood (Wajant et al., 2005; Wilson et al., 2009). It is noteworthy that the chemotherapy-induced apoptosis resistance (secondary or acquired apoptosis resistance) has a severely detrimental impact on chemotherapy because it not only dampens the anticancer activity of the drugs, but it also promotes cancer progression. For example, when TNF-related apoptosis-inducing ligand (TRAIL) loses its cell-killing capacity, it promotes proliferation and metastasis in apoptosis-resistant cancer cells (Malhi and Gores, 2006). Therefore, it is crucial to understand the mechanism of acquired apoptosis resistance to retain the cancer-killing activity while circumventing the cancer-promoting potential of chemotherapeutics.

Apoptosis plays a major role in preventing normal cellular integrity and is strictly regulated. Two main distinct apoptosis pathways have been developed, namely the intrinsic and extrinsic pathways (Heath-Engel et al., 2008; Papenfuss et al., 2008). Initiating signals in the intrinsic pathway are

ABBREVIATIONS: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Adr, doxorubicin; CDDP, cisplatin; COX-2, cyclooxygenase-2; c-FLIP, cellular FLICE-like inhibitory protein; DR, death receptor; PC, parental cell; TR, tumor necrosis factor-related apoptosis-inducing ligand-resistant; TNF, tumor necrosis factor; siRNA, small interfering RNA; IKK, IκB kinase complex; PARP, poly(ADP-ribose) polymerase; LDH, lactate dehydrogenase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; GSK3β, glycogen synthase kinase 3β; z-VAD, N-benzyloxycarbonyl-Val-Ala-Asp-; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride.

generated by developmental cues or cellular damage that cause the loss of mitochondrial potential and release of proapoptotic factors such as cytochrome c and Smac from the mitochondria to the cytosol. A protein complex called apoptosome consisting of cytochrome c and Apaf1 subsequently is formed to activate the initiator caspase-9, which activates effector caspases 3 and 7 that execute apoptosis. This pathway involves the physical and functional interplay between the prosurvival Bcl2 family members, including Bcl2, Bcl-XL, and Mcl-1, and the proapoptosis members Bax, Bak, and Bok. The extrinsic pathway is activated by stimulation from outside of the cell through the ligation of the TNF family of cytokines to their cognate receptors located on the cell membrane. The TNF family of receptors are also called death receptors and include TNF's TNF receptor 1 and TRAIL's death receptors 4 and 5 (DR4 and DR5). This pathway is initiated by the formation of the death-inducing signaling complex consisting of the receptor, receptor-interacting protein, and Fas-associated death domain that activates initiator caspase-8, which leads to activation of effector caspases 3 and 7 to execute apoptosis. The caspase-8 competitor cellular FLICE-like inhibitory protein (c-FLIP) can be recruited to the death-inducing signaling complex to inhibit the recruitment and activation of caspase-8 (Ashkenazi, 2008). It is noteworthy that cross-talks between the two apoptosis pathways occur to accelerate cell death. For example, the extrinsic pathway-activated caspase-8 cleaves Bid, a BH3-only member of the Bcl-2 family, to generate the proapoptotic tBid that migrates to mitochondria and activates the mitochondrial apoptosis pathway (Papenfuss et al., 2008; Wang and Lin, 2008). In addition, there is a positive feedback loop that leads to further activation of the initiator caspases by effector caspases (Pop and Salvesen, 2009). Regarding anticancer agent-induced cytotoxicity, TRAIL mainly activates the extrinsic pathway, whereas the DNA-damaging drugs doxorubicin [Adriamycin (Adr); Bedford Laboratories, Bedford, OH] and cisplatin (CDDP) mainly induce the intrinsic pathway (Wilson et al., 2009).

We have recently established acquired resistance to TRAIL-induced apoptosis in lung cancer cell lines by continuously exposing the TRAIL-sensitive lung cancer cells to a nontoxic dose and gradually increasing the concentrations of TRAIL. We found that the Akt-dependent overexpression of c-FLIP$_L$ and Mcl-1$_L$ is associated with acquired TRAIL resistance (Wang et al., 2008). In this study, we further determined that TRAIL-resistant cancer cells are also refractory to apoptosis caused by other anticancer therapeutic drugs, which is associated with an Akt-mediated increase of cyclooxygenase-2 (COX-2) expression. Pharmacological or genetic suppression of COX-2 drastically alleviated apoptosis resistance accompanied by reduction in Mcl-1 expression. These results established a novel pathway composed of Akt, COX-2, and Mcl-1 that confers lung cancer cells' acquired resistance to apoptosis. This pathway could provide a molecular target for preventing and alleviating acquired apoptosis resistance during anticancer chemotherapy.

Materials and Methods

Reagents. Glutathione transferase TRAIL was prepared as described previously (Lin et al., 2000; Chen et al., 2007b). Adr and CDDP were from Sigma-Aldrich (St. Louis, MO). IKK inhibitor II, COX-2 inhibitor II, and LY294002 were purchased from Calbiochem (San Diego, CA). Small interfering RNA (siRNA; SMATpool) for COX-2, Mcl-1, Akt (pooled siRNAs that targeting the Akt isoforms Akt1, Akt2, and Akt3) and negative control were purchased from Dharmacon (Lafayette, CO). The following antibodies were used for Western blot: anti-caspase-8 and anti-caspase-3 (BD Biosciences Pharmingen, San Diego, CA); anti-poly(ADP-ribose) polymerase (PARP) (BioSource International, Camarillo, CA); anti-β-tubulin, anti-β-actin (Sigma-Aldrich); anti-Akt and -phospho-Akt(Ser473) and -phospho-GSK3β (Cell Signaling Technology, Danvers, MA); anti-COX-2 (Cayman Chemical, Ann Arbor, MI); and anti-c-FLIP and anti-Mcl-1 (Alexis Laboratories, San Diego, CA).

Cell Culture, Transfection, and Establishing the TRAIL-Resistant Cells. The human lung cancer cell lines H460 and H1568 were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mM glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin. For transfection of siRNA, cells were grown in 12-well plates and were transfected with siRNA with INTERFERin siRNA transfection reagent according to manufacturer's instructions (Polyplus-transfection, Inc. New York, NY). The knockdown of protein expression was determined by Western blot. H460-TRAIL-resistant (TR) and H1568-TR cells were established by continuously exposing the TRAIL-sensitive H460 and H1568 cells to increasing doses of TRAIL as described previously (Wang et al., 2008). The parental cell (PC) lines were designated as H460-PC and H1568-PC, respectively.

Cytotoxicity Assay. Cytotoxicity was determined using a lactate dehydrogenase (LDH) release-base cytotoxicity detection kit (Promega, Madison, WI). Cells were seeded in 48-well plates at 70 to 80% confluence, cultured overnight, and then treated as indicated in the figure legends. LDH release was determined, and cell death was calculated as described previously (Wang et al., 2006b; Ju et al., 2007). The experiments were repeated no less than three times, and representative results are shown in the figures.

Western Blotting. Total protein lysates were prepared by suspending cells in M2 buffer (20 mM Tris-HCl, pH 7.6, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 μg/ml leupeptin). Equal amounts of cell lysate were resolved by 10, 12, or 15% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted with the RNAeasy kit (QIAGEN, Valencia, CA). One microgram of RNA from each sample was used as a template for cDNA synthesis with a reverse transcription kit (Promega). An equal volume of cDNA product was used in the PCR. The primers used were the following: COX-2, CGCTCAGCCATACAGCAAATCCTT and GTGCACTGTGTTTGGAGTGGGTTT; and β-actin, CCAGCCTTCCTTCCTGGGCAT and AGGAGCAATGATCTTGATCTTCATT. The reaction condition was 94°C for 45 s, 55°C for 40 s, and 72°C for 45 s. For COX-2 and β-actin, the cycles for PCR were 27 and 21, respectively. PCR products were run on 2% agarose gel with 0.5 μg/ml ethidium bromide, visualized, and photographed.

Tumorigenicity Assay in Nude Mice. H460-PC and H460-TR cells were harvested and washed twice with phosphate-buffered saline. Aliquots of 1 million cells were suspended in 50 μl of phosphate-buffered saline mixed with 50 μl of Matrigel (BD Biosciences, San Jose, CA) and injected subcutaneously at the right and left flanks of athymic nude mice (BALB/cnu/nu, 4–6 weeks old). When the average tumor volume reached approximately 100 mm^3, the mice injected with H460-PC or H460-TR cells were randomized into three groups of three mice per group. Group 1 was the untreated control, group 2 was treated with TRAIL (15 mg/kg, i.p.), and group 3 was treated with CDDP (6 mg/kg, i.p.) on the indicated days. Tumors were

measured using a caliper, and tumors were calculated using the following formula: tumor volume = length × width²/2.

Statistics. Data are expressed as mean ± S.D. Statistical significance was examined by one-way analysis of variance. In all analyses, $P < 0.05$ was considered statistically significant.

Results

Acquired Apoptosis-Resistance Established in H460 and H1568 Cells by Long-Term Exposure to TRAIL. We generated acquired TRAIL-resistant cells by continuously exposing the ensured TRAIL-sensitive H460 and H1568 cells to gradually increasing concentrations of TRAIL, which was started with a nontoxic low dose and gradually increased to a high dose that completely killed the parental cells (Wang et al., 2008). The resulting cell lines, designated as H460-TR and H1568-TR, respectively, acquired resistance to TRAIL-induced apoptosis (Fig. 1, A and B) (Wang et al., 2008). The TRAIL-resistant cells also acquired cross-resistance to the anticancer drugs Adr- and CDDP-induced cytotoxicity (Fig. 1, A and B). The cytotoxicity induced by TRAIL, Adr, and CDDP was confirmed to be mainly apoptotic because it was associated with caspase activation and cytochrome *c* release and could be effectively inhibited with the pan-caspase inhibitor z-VAD (Fig. 1C and data not shown). The resistance to TRAIL- or CDDP-induced cancer cell death was further confirmed in vivo with the H460 parental cells (H460-PC)

and H460-TR cells and a xenografted tumor nude mouse model (Fig. 1D). TRAIL or CDDP treatment caused substantial growth retardation in tumors derived from H460-PC cells (Fig. 1D, right). However, the H460-TR cells were complete refractory to these drugs (Fig. 1D, left). Tumors formed by H460-TR cells grew slower than those formed by H460-PC cells, which is consistent with previous reports showing that chemoresistant cancer cells grow more slowly than sensitive ones (Kusumoto et al., 1996; Gatenby et al., 2009). Taken together, these in vitro and in vivo results suggest that the continuously TRAIL-exposed cells have acquired resistance against apoptosis mediated by both the extrinsic and intrinsic pathways, mainly induced by TRAIL, and Adr and CDDP, respectively.

COX-2 Expression Is Dramatically Increased in H460-TR and H1568-TR Cells. Gene expression microarray was used to search for potential factors contributing to the acquired apoptosis resistance in H460-TR cells, and COX-2 was one of the genes most significantly increased in these cells. To verify this result, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot were used to detect COX-2 mRNA and COX-2 protein, respectively. COX-2 was barely expressed in H460 cells. However, both the COX-2 mRNA and protein expression levels were significantly higher in H460-TR cells (Fig. 2A). A similar observation was made in H1568 parental cells (H1568-PC)
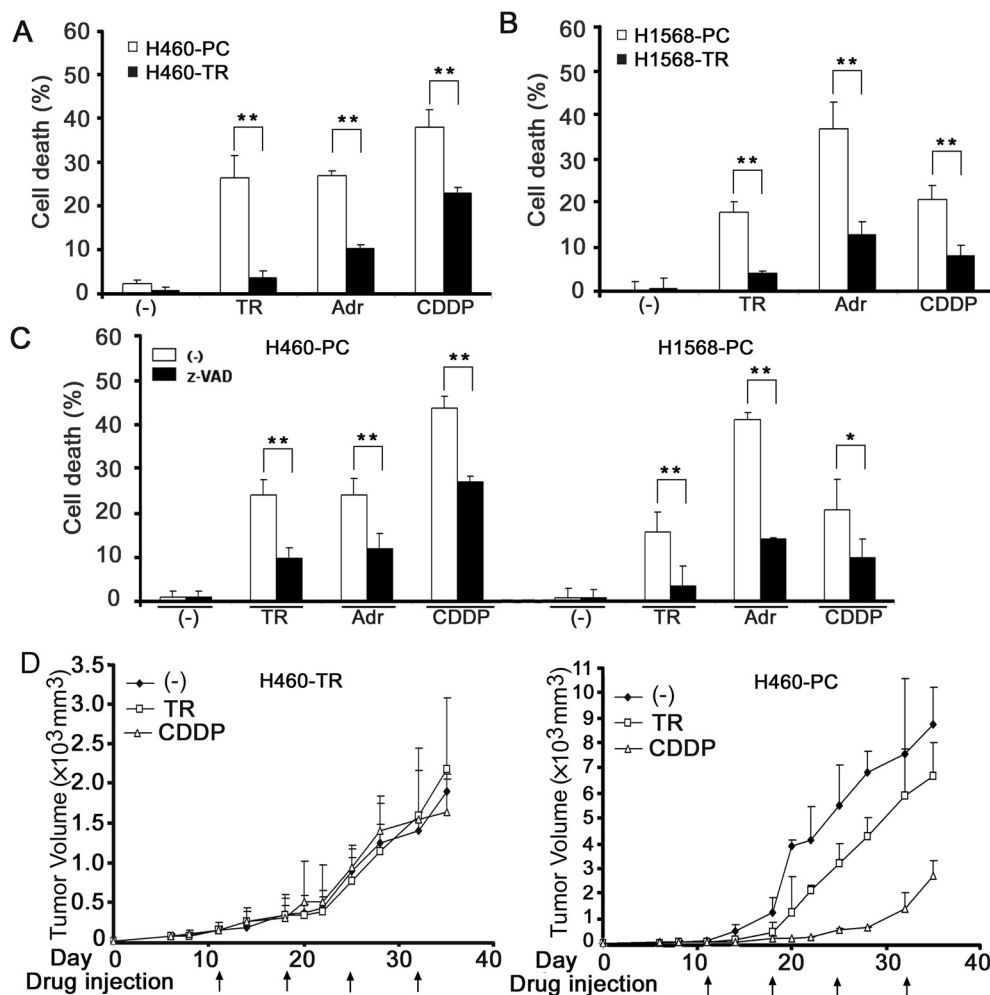

Fig. 1. Acquired apoptosis resistance established in H460 and H1568 cells by long-term TRAIL exposure. A, H460-PC and H460-TR cells were treated with TRAIL (50 ng/ml), Adr (0.25 μg/ml), or CDDP (50 μM) for 30 h. Cell death was measured by LDH leakage assay. Data shown are the mean ± S.D.; *, $p < 0.05$; **, $p < 0.01$. B, H1568-PC and H1568-TR cells were treated with TRAIL (100 ng/ml), Adr (0.5 μg/ml), or CDDP (100 μM) for 30 h. Cell death was detected as described in A. C, H460-PC and H1568-PC cells were pretreated with z-VAD (10 μM) or were left untreated for 1 h followed by TRAIL (50 ng/ml for H460-PC, 100 ng/ml for H1568-PC), Adr (0.25 μg/ml for H460-PC, 0.5 μg/ml for H1568-PC), or CDDP (50 μM for H460, 100 μM for H1568-PC) for 30 h. Cell death was detected as described in A. D, H460-PC or H460-TR cells (10^6 in 50 μl) were injected subcutaneously at the right and left flanks of athymic nude mice to form tumors. The mice were treated with TRAIL (15 mg/kg) or CDDP (6 mg/kg) at the indicated times or were left untreated. Tumor volume was detected and expressed as mean ± S.D.

and H1568-TR cells (Fig. 2B). Because COX-2 was reported to be involved in cancer cells' insensitivity to apoptosis (Brown and DuBois, 2004), these results prompted us to investigate whether COX-2 plays a role in acquired apoptosis resistance generated by long-term TRAIL exposure.

Suppressing COX-2 Substantially Sensitizes H460-TR and H1568-TR cells to TRAIL-, Adr-, and CDDP-Induced Apoptosis. A COX-2 inhibitor was used first to examine the effect of suppressing COX-2 activity on TRAIL-induced cytotoxicity. The results showed that the COX-2 inhibitor substantially sensitized H460-TR cells to TRAIL-induced cytotoxicity (Fig. 3A). It is interesting that this

COX-2 inhibitor had a marginal effect on TRAIL-induced cytotoxicity in H460-PC cells expressing very low levels of COX-2 protein (Fig. 3A). Likewise, the COX-2 inhibitor significantly sensitized H1568-TR but not H1568-PC cells to TRAIL-induced cytotoxicity (data not shown). These results strongly suggest that the potentiation of cytotoxicity by the COX-2 inhibitor is COX-2-specific, which differs from reports that some COX-2 inhibitors sensitized cancer cell apoptosis in a COX-2-independent manner (Chen et al., 2007a; Chuang et al., 2008). Likewise, the COX-2 inhibitor also markedly potentiated Adr- or CDDP-induced cytotoxicity (Fig. 3B).

To validate and substantiate the results from the COX-2 inhibitor, COX-2 siRNA was used to deplete COX-2 protein from the cells. COX-2 siRNA significantly potentiated TRAIL-induced apoptosis in H460-TR but not H460-PC cells (Fig. 4A). COX-2 expression was effectively suppressed by
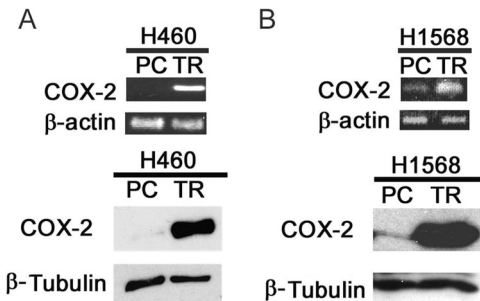
Fig. 2. COX-2 expression is increased in H460TR and H1568-TR cells. A, top, equal amounts of total RNA from H460-PC and H460-TR cells were detected for COX-2 mRNA expression by RT-PCR. β-Actin was detected as an input control. Bottom, equal amounts of cell extracts from H460-PC and H460-TR cells were detected for COX-2 protein expression by Western blot. B, top, equal amounts of total RNA from H1568-PC and H1568-TR cells were detected for COX-2 mRNA expression by RT-PCR. β-Actin was detected as an input control. Bottom, equal amounts of cell extracts from H1568-PC and H1568-TR cells were detected for COX-2 protein expression by Western blot. β-Tubulin was detected as an input control.
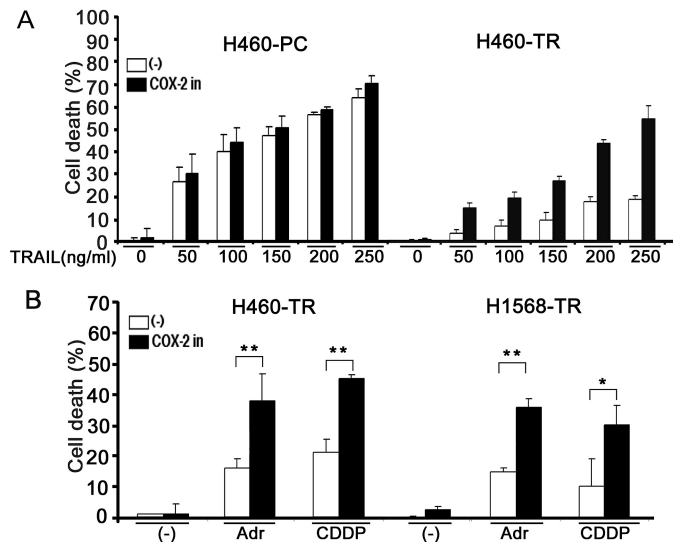
Fig. 3. COX-2 inhibitor sensitizes H460-TR and H1568-TR cells to TRAIL-, Adr-, and CDDP-induced apoptosis in vitro. A, H460-PC and H460-TR cells were pretreated with COX-2 inhibitor II (100 nM) for 1 h or were left untreated followed by the indicated concentrations of TRAIL for 30 h. Cell death was measured by LDH leakage assay. Data shown are the mean $\pm$ S.D. B, H460-TR or H1568-TR cells were pretreated with COX-2 inhibitor II (100 nM) for 1 h or were left untreated followed by exposure to Adr (0.25 μg/ml for H460-TR, 0.5 μg/ml for H1568-TR) or CDDP (25 μM for H460-TR, 50 μM for H1568-TR) for 30 h. Cell death was detected as described in A; $*$, $p < 0.05$; $**$, $p < 0.01$.
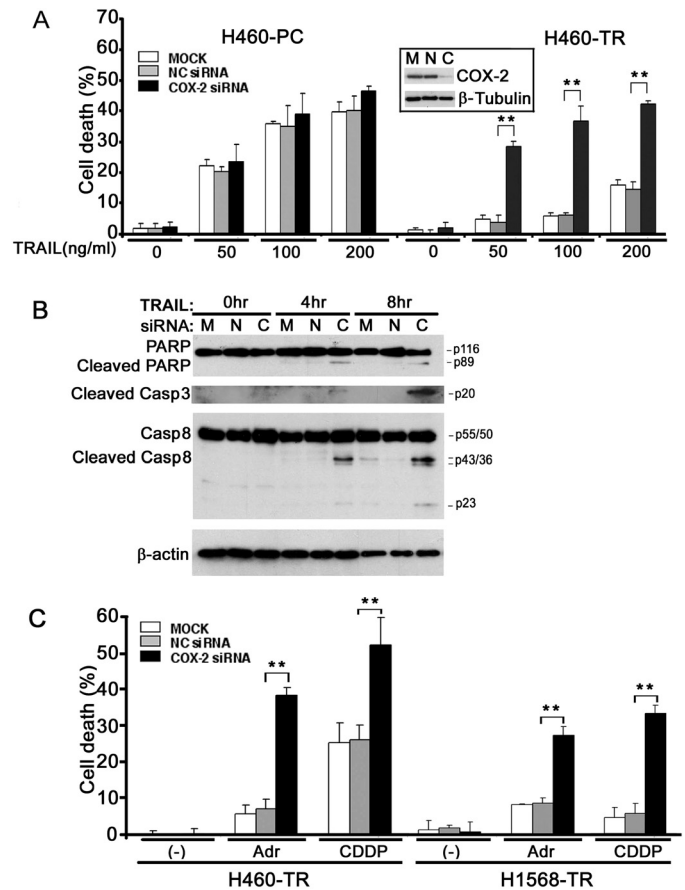

Fig. 4. Knockdown of COX-2 potentiated TRAIL-, Adr-, and CDDP-induced apoptosis. A, H460-PC and H460-TR cells were mock-transfected or were transfected with 5 nM COX-2-siRNA or negative control siRNA. Forty-eight hours after transfection, the cells were treated with the indicated concentrations of TRAIL for 30 h or were left untreated. Cell death was measured by LDH leakage assay. Data shown are mean $\pm$ S.D.; $**$, $p < 0.01$. Inset, knockdown of COX-2 was confirmed by Western blot in H460-TR cells. B, H1568-TR cells were mock-transfected or were transfected with 5 nM COX-2-siRNA or negative control siRNA. Forty-eight hours after transfection, the cells were treated with TRAIL (100 ng/ml) for 4 or 8 h or were left untreated (0 h). PARP and caspases 3 and 8 were detected by Western blot. β-Actin was detected as an input control. C, H460-TR or H1568-TR cells were mock-transfected or were transfected with 5 nM COX-2-siRNA or NC siRNA. Forty-eight hours after transfection, the cells were treated with Adr (0.25 μg/ml for H460-TR, 0.5 μg/ml for H1568-TR) or CDDP (50 μM for H460-TR and 100 μM for H1568-TR) for 30 h or were left untreated. Cell death was detected as described in A. $**$, $p < 0.01$. C, COX-2 siRNA; M, mock transfection; N, negative control siRNA.

the COX-2 siRNA (Fig. 4A, inset). The effect was specific to COX-2 depletion because the transfection of control negative siRNA, which did not alter the expression of COX-2, had no effect on TRAIL-induced cell death (Fig. 4A). The sensitized cytotoxicity was mainly apoptotic because it was associated with enhanced activation of caspases 8 and 3 and cleavage of the caspase 3 substrate PARP (Fig. 4B). Similar effects were seen in H1568-PC and H1568-TR cells (data not shown). In addition, Adr- or CDDP-induced cytotoxicity was also substantially increased in both H460-TR and H1568-TR cells when COX-2 siRNA, but not the control negative siRNA, was transfected (Fig. 4C). These results imply that COX-2 over-expression plays a substantial role in acquired apoptosis resistance.

COX-2 Expression in H460TR and H1568 TR Cells Is Regulated by Akt. Our previous studies found that Akt-mediated overexpression of c-FLIP$_L$ and Mcl-1$_L$ plays an important role in acquired TRAIL-resistance (Wang et al., 2008). Akt was constitutively activated in H460-TR and H1568-TR cells (Fig. 5A). Previous reports suggested that under some circumstances, the PI3K/Akt pathway regulates COX-2 expression (Ladu et al., 2008). Thus, we examined whether the COX-2 overexpression in H460-TR and H1568-TR cells is due to constitutive Akt activation. When Akt activity was suppressed with the PI3K/Akt inhibitor LY294002, COX-2 mRNA and protein expression was dramatically inhibited in H460-TR cells (Fig. 5B). In contrast, the IKK inhibitor II, which is potent in suppressing NF-κB (Bai et al., 2009), had no detectable effect on COX-2 expression (Fig. 5, B and C). These results were validated with Akt siRNAs that target all of the Akt isoforms (Akt1, Akt2, and Akt3), which effectively reduced the expression and activity of Akt, that suppressed COX-2 expression (Fig. 5D). As expected, the activity of GSK3β was also suppressed by the Akt siRNA (Fig. 5D). In contrast, suppressing COX-2 with COX-2 siRNA had no de-

tectable effect on the activity of Akt and GSK3β (Fig. 5E). These results demonstrate that COX-2 functions downstream of Akt, and the elevated Akt activity is pivotal for COX-2 overexpression in the apoptosis-resistant cells.

COX-2 Regulates Mcl-1 Expression and Mcl-1-Mediated Apoptosis Resistance in H460-TR and H1568-TR Cells. Our previous studies found that Akt-mediated overexpression of c-FLIP$_L$ and Mcl-1$_L$ plays an important role in acquired TRAIL resistance (Wang et al., 2008). Because in some cell contexts COX-2 expression regulates the expression and function of c-FLIP or Mcl-1 (Lin et al., 2001; Xu et al., 2004), we examined whether the up-regulation of c-FLIP$_L$ or Mcl-1$_L$ expression underlies the mechanism of COX-2-mediated apoptosis resistance. The COX-2 expression level was significantly reduced in H460-TR cells 24 h after transfection with COX-2 siRNA, whereas the expression of Mcl-1$_L$ was dramatically decreased starting at 48 h. The reduction of Mcl-1$_L$ expression was specific to COX-2 siRNA transfection because the transfection of negative siRNA did not affect Mcl-1$_L$ expression (Fig. 6A, top). Similar effects were seen in the H1568-TR cells (Fig. 6A, bottom). The slower kinetics of Mcl-1$_L$ reduction compared with those of COX-2 further suggest that the decrease in Mcl-1$_L$ was a secondary effect of COX-2 suppression. In contrast, the expression of c-FLIP$_L$ was not affected by COX-2 siRNA in either H460-TR or H1568-TR cells (Fig. 6A), suggesting that c-FLIP$_L$ is not involved in COX-2-mediated apoptosis resistance. The knockdown of Mcl-1$_L$ with siRNA had no detectable effect on expression of COX-2, p-Akt, or p-GSK3β, further indicating that Mcl-1$_L$ is downstream of Akt and COX-2 (Fig. 6B). In both H460-TR and H1568-TR cells, Mcl-1 siRNA, but not the negative control siRNA, dramatically sensitized TRAIL-, Adr-, and CDDP-induced cytotoxicity, suggesting that Mcl-1$_L$ is one of the main downstream effectors of COX-2 in mediating the acquired apoptosis resistance (Fig. 6C).
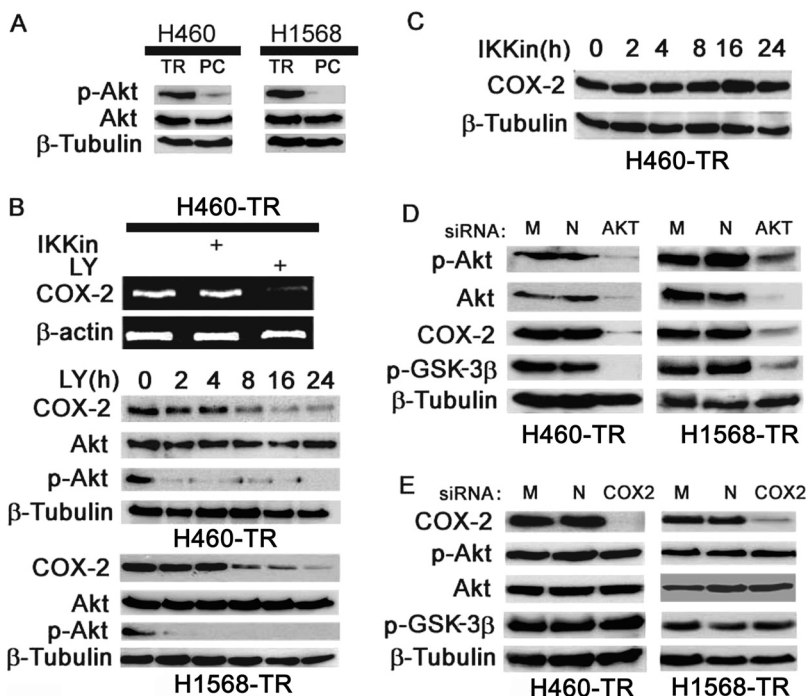
Fig. 5. Suppressing Akt reduces COX-2 expression in H460-TR and H1568-TR cells. A, equal amounts of cell extract from the indicated cells were Western-blotted for p-Akt and total Akt. β-Tubulin was detected as an input control. B, top, H460-TR cells were treated with IKK inhibitor II (10 nM) or LY294002 (10 μM) overnight. Expression of COX-2 mRNA was detected by RT-PCR. β-Actin was detected as an input control. Center and bottom, H460-TR and H1568-TR cells were treated with LY294002 for the indicated times. COX-2, Akt, and p-Akt were detected by Western blot. C, H460-TR cells were treated with IKK inhibitor II for the indicated times. COX-2 was detected by Western blot. D, H460-TR and H1568-TR cells were mock-transfected or were transfected with 5 nM Akt- or negative control siRNA. Forty-eight hours after transfection, COX-2, Akt, and p-Akt were detected by Western blot. β-Tubulin was detected as an input control. E, H460-TR and H1568-TR cells were mock-transfected or transfected with 5 nM COX-2- or negative control siRNA. Forty-eight hours after transfection, COX-2, p-Akt, and p-GSK3β were detected by Western blot. β-Tubulin was detected as an input control. M, mock-transfected; N, negative control.
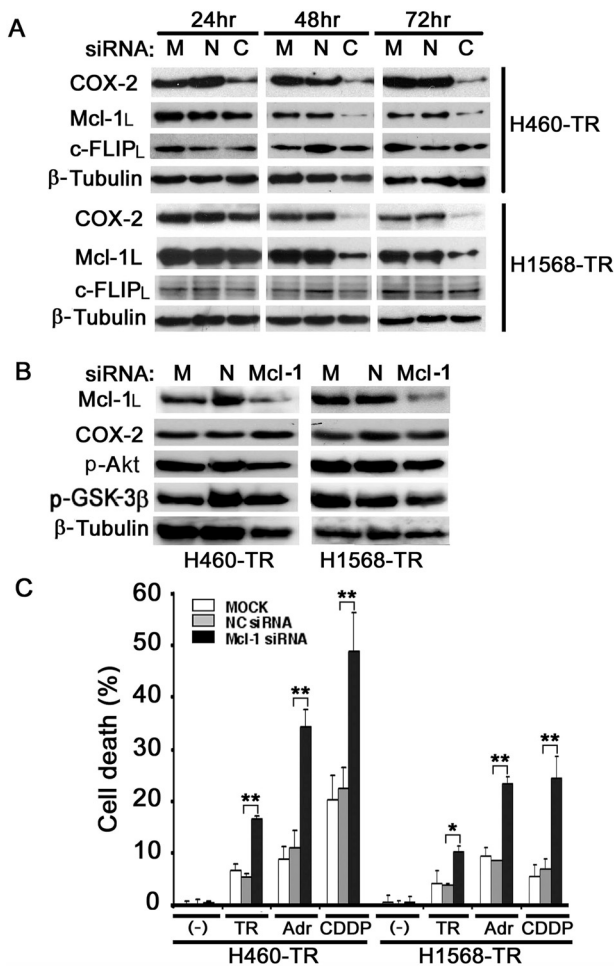
Discussion

This study establishes a new cellular signaling pathway consisting of Akt, COX-2, and Mcl-1$_L$ that confers acquired apoptosis resistance to lung cancer cells. Having discovered that Akt and Mcl-1$_L$ play an important role in acquired resistance against TRAIL-induced apoptosis (Wang et al., 2008), we now have identified that a dramatic overexpression of COX-2 in lung cancer cells is integral to acquired apoptosis resistance that can be abrogated through inhibiting Akt. Suppressing COX-2 activity with either a chemical inhibitor or COX-2 siRNA drastically alleviated resistance to therapeutic-induced apoptosis. Alleviating this apoptosis resistance was associated with a reduction in Mcl-1$_L$ expression. Thus, our data suggest that Akt, COX-2, and Mcl-1$_L$ form a

Fig. 6. COX-2 regulates Mcl-1 expression and Mcl-1-mediated apoptosis resistance in H460-TR and H1568-TR cells. A, H460-TR and H1568-TR cells were mock-transfected or were transfected with 5 nM COX-2- or negative control siRNA. COX-2, Mcl-1$_L$, and c-FLIP$_L$ were detected by Western blot 24, 48, or 72 h after transfection. β-Tubulin was detected as an input control. B, H460-TR and H1568-TR cells were mock-transfected or were transfected with 5 nM Mcl-1- or negative control siRNA. Mcl-1$_L$, COX-2, p-Akt, and p-GSK3β were detected by Western blot 48 h after transfection. β-Tubulin was detected as an input control. C, H460-TR and H1568-TR cells were mock-transfected (□) or were transfected with 5 nM Mcl-1-siRNA (■) or negative control siRNA (▨). Forty-eight hours after transfection, H460-TR cells were treated with TRAIL (50 ng/ml), Adr (0.25 μg/ml), or CDDP (50 μM), and H1568-TR cells were treated with TRAIL (100 ng/ml), Adr (0.5 μg/ml), or CDDP (100 μM) for 30 h. Cell death was measured by LDH leakage assay. Data shown are the mean $\pm$ S.D. $*$, $p < 0.05$; $**$, $p < 0.01$. C, COX-2; N, negative control; M, mock-transfected.

novel signaling pathway conferring lung cancer cells' acquired apoptosis resistance, and this pathway could be a molecular target for preventing and circumventing acquired chemoresistance in lung cancer.

As a potent mechanism for chemoresistance, acquired apoptosis resistance contributes to therapy failure and tumor recurrence (Wilson et al., 2009). The mechanism of acquired apoptosis resistance is complex and not well elucidated (Fulda, 2009; Wilson et al., 2009). Activating the drug inactivation or clearance mechanisms can directly remove apoptosis-inducing drugs from cancer cells. For example, chemotherapy induces the expression of the ATP-binding cassette multidrug transporter proteins that trigger efflux of the drugs. Directly suppressing the apoptosis pathways or reducing the activity of the apoptosis machinery is also involved (Fulda, 2009). In addition, activating antiapoptotic cell survival signals such as NF-κB and Akt, which have been commonly found in a variety of cancer cells, could be another mechanism in acquired apoptosis resistance. Our results show a constitutive Akt activation in acquired apoptosis resistance, adding more evidence supporting the involvement of cell survival signaling, although other mechanisms are not excluded.

As a master kinase, Akt regulates apoptosis through directly phosphorylating the apoptotic machinery proteins such as caspase-9 and apoptosis regulator BAD and indirectly suppressing apoptosis through modulating other pathways such as NF-κB, c-Jun NH$_2$-terminal kinase, and p53 (Hanada et al., 2004; Wang et al., 2007). In this report, we show that Akt activates a COX-2-mediated antiapoptotic pathway that involves the antiapoptotic Bcl2 family member Mcl-1. The expression of COX-2 at both the messenger RNA and protein levels was effectively blocked when Akt was suppressed with either siRNA or a chemical inhibitor, suggesting that COX-2 is a downstream factor of Akt, and the up-regulation of COX-2 is probably due to increased transcription rate or COX-2 mRNA stability. This is consistent with previous reports showing that PI3K/Akt regulates COX-2 expression (Ladu et al., 2008; Lee et al., 2008b). Numerous cell signaling pathways, including NF-κB, Ap1, and PI3K/Akt, have been reported to be involved in inducing COX-2 expression (Telliez et al., 2006). Under some circumstances NF-κB also is downstream of Akt (Ozes et al., 1999; Wang et al., 2007). However, NF-κB is unlikely to be involved in up-regulation of COX-2 by Akt in the apoptosis-resistant cells established in this study because blocking NF-κB had no detectable effect on this action of Akt. Because we clearly detected a reduction of GSK3β activity when Akt was blocked and GSK3β was implicated in regulating COX-2 expression (Rao et al., 2004; Wang et al., 2006a), it remains to be determined whether the Akt downstream kinase GSK3β is involved in regulating COX-2 expression in the context of acquired apoptosis resistance.

COX-2 is inducible by inflammatory cytokines, tumor promoters, and growth factors, and thus could be involved in inflammation-associated cancer development (Greenhough et al., 2009). Indeed, increased expression of COX-2 is found in a variety of cancer cells that are resistant to apoptosis (Greenhough et al., 2009). Therefore, COX-2 is implicated as a target for alleviating apoptosis resistance in cancer cells. COX-2 inhibitors were tested for cancer therapy, and substantial anticancer activity was achieved. However, because

of cellular effects that are unrelated to COX-2 activity, such as inducing DR5 or triggering endoplasmic reticulum stress that directly contributes to COX-2-inhibitor-induced apoptosis (Kern et al., 2006; Chen et al., 2007a), some questions have been raised regarding the role of COX-2 in apoptosis regulation. Here we show that suppressing COX-2 activity with chemical inhibitors and eliminating COX-2 protein with siRNA were equally effective in sensitizing the cells to apoptosis induced by different anticancer agents and was not accompanied by increases in DR5 (data not shown) as reported previously (Chen et al., 2007a). The difference in non-COX-2-related activity between our findings and those of others may be due to the use of different COX-2 inhibitors (Hardy et al., 2003; Malhotra et al., 2004). Nevertheless, our data clearly show that COX-2 has an antiapoptosis role in cancer cells with acquired apoptosis resistance.

How COX-2 converts cells to be apoptosis resistance is not well elucidated. In this study, we found that COX-2 is mainly involved in Mcl-1$_L$- but not c-FLIP$_L$-mediated antiapoptotic signaling. Because c-FLIP$_L$ is clearly involved in Akt-mediated resistance to TRAIL-induced apoptosis (Wang et al., 2008), this finding suggests that up-regulation of COX-2 underlies one of the mechanisms of Akt-mediated apoptosis resistance. Because Mcl-1 messenger RNA is not increased in H460-TR and H1568-TR cells, the regulation of Mcl-1 expression by COX-2 is probably through the stability of Mcl-1$_L$ protein (Wang et al., 2008). It remains to be determined how COX-2 stabilizes Mcl-1$_L$ to ensue apoptosis resistance. It is unlikely that the proapoptosis Bcl-2 family member Noxa is involved in COX-2-mediated stabilization of Mcl-1, because knockdown of COX-2 had no effect on Noxa expression (data not shown). In addition, it was not clear why suppressing COX-2 enhanced TRAIL-induced activation caspase-8, the initiator caspase for the extrinsic apoptosis pathway (Fig. 4B), but had no effect on c-FLIP$_L$ expression (Fig. 6A). It is possibly due to the cross-talk between the intrinsic and extrinsic pathways that is regulated by Mcl-1(Pop and Salvesen, 2009). Indeed, Mcl-1 was able to block caspase-8 activation through the intrinsic pathway (Keuling et al., 2009).

It is noteworthy that although COX-2 was overexpressed in ~70% of human lung tumors, heterogeneity (positive and negative) of COX-2 expression was commonly seen in these tumors (Hida et al., 1998; Lee et al., 2008a). Thus, it is interesting to determine whether the Akt/COX-2/Mcl-1 pathway is involved in constitutive COX-2 expression and response to chemotherapy in COX-2-expressing tumor cells. It is also warranted to determine whether this pathway is activated in COX-2-negative tumor cells and whether the activation of this pathway is associated with tumor relapse during chemotherapy. Several clinical trials combining the COX-2 inhibitor celecoxib and therapeutics such as paclitaxel, etoposide, and cisplatin showed moderate potentiated effects (Arúajo et al., 2009; Mutter et al., 2009; Suzuki et al., 2009). However, it was noted that the responders had either low COX-2 activity or higher efficacy of COX-2 inhibition by the treatment, suggesting that to decrease the COX-2 expression or activity to lower than the responding threshold could be an important determinant for cancer patients' response (Mutter et al., 2009). It would be interesting to determine whether acquired apoptosis associated with activation of the Akt/COX-2/Mcl-1 pathway is induced in the patients with poor response. Nevertheless, developing more effective and cancer cell-specific COX-2-inhibiting approaches is warranted for cancer therapy.

In summary, results from this study demonstrate a new cellular signaling pathway consisting of Akt, COX-2, and Mcl-1, established during long-term TRAIL exposure that renders lung cancer cells resistant to therapeutic-induced apoptosis. The key components of this pathway could be molecular targets for circumventing acquired chemoresistance in lung cancer. Further study is warranted to understand how this pathway is constitutively activated during the development of acquired apoptosis resistance.

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