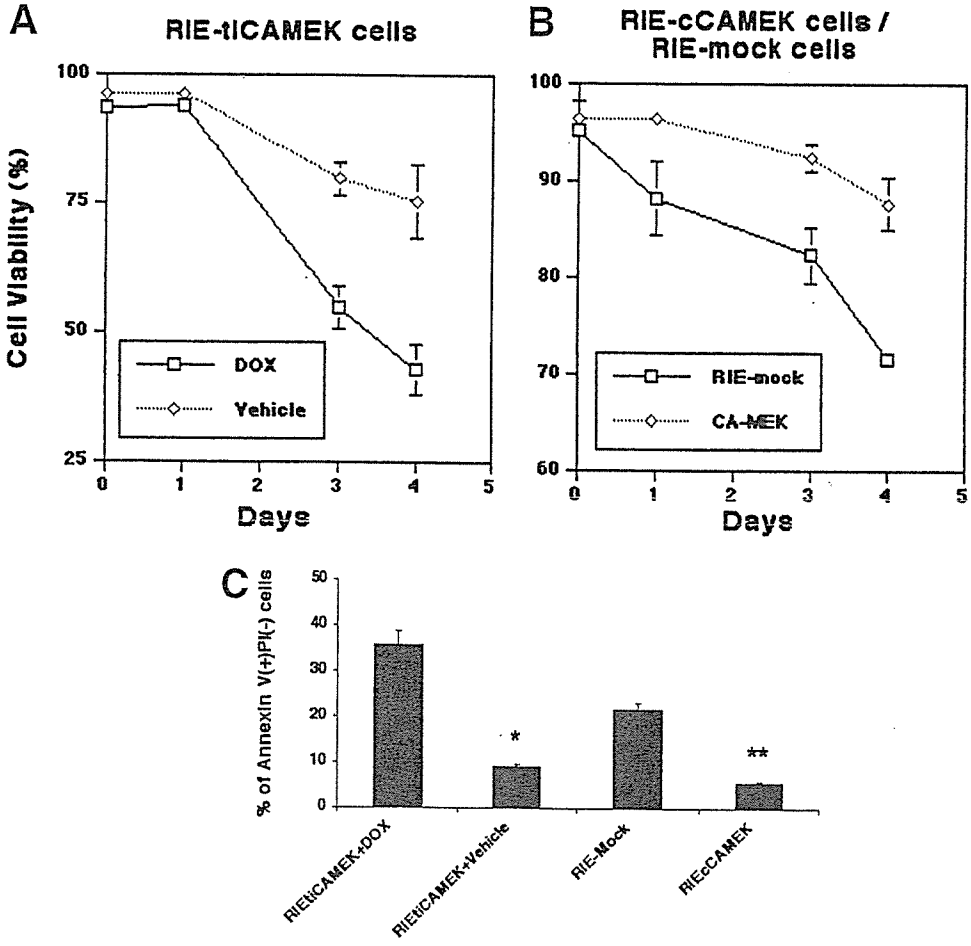

Figure 2. CA-MEK cells are resistant to NaB-induced programmed cell death. (*A*) RIE-tiCAMEK cells were treated with 2 μg/mL DOX (no CA-MEK expression) or vehicle (CA-MEK expression) for 48 hours. Then the media was replaced with 5 mmol/L NaB plus 2 μg/mL DOX or vehicle, and both floating and attached cells were counted at the indicated times. (*B*) RIE-Mock and RIE-cCAMEK cells were treated with 5 mmol/L NaB, and both floating and attached cells were counted at the indicated times. Values are the means ± SE of 3 separate experiments performed in triplicate. (*C*) The percentage of Annexin V–positive, propidium iodide (PI)-negative cells. Values are the means ± SE of 3 separate experiments performed in triplicate. $*P = .0001$, $**P < .0001$ compared with control cells (RIE-tiCAMEK + DOX cells or RIE-Mock cells).

MEK Signaling Suppresses NaB-Mediated Apoptosis

To evaluate the role of MEK-ERK signaling on intestinal epithelial programmed cell death after terminal differentiation, we induced apoptosis in RIE-1 cells by treatment with NaB. RIE-1 cells do not spontaneously differentiate in culture[39] but will undergo partial differentiation and cell death following treatment with NaB.[34] For this purpose, we also generated CA-MEK–expressing RIE-1 cells under the control of the Tet-Off gene expression system[50] (RIE-tiCAMEK). The Tet-Off system allows for tighter control and reproducibility of transgene expression. RIE-tiCAMEK cells were treated with DOX (2 μg/mL), which represses transgene expression. The cells treated with DOX (no CA-MEK expression) showed a notable decrease of cell viability following exposure to NaB, whereas vehicle-treated cells (CA-MEK expression) showed a marked resistance to cell death (Figure 2*A*). Similar differences in cell viability were also observed between RIE-Mock and RIE-cCAMEK cells (Figure 2*B*). In control experiments, we found that the concentration of DOX did not alter cell growth or cell viability in parental RIE-1 cells (data not shown), indicating that the results were not due to a nonspecific effect of DOX. Additionally, CA-MEK–expressing RIE cells did not differ in their growth rate when compared with controls (data not shown).

One characteristic of apoptotic cells is the loss of plasma membrane asymmetry, resulting in the exposure of phosphatidylserine residues at the outer plasma membrane leaflet.[51] Annexin V interacts strongly and specifically with phosphatidylserine and can be used as a surrogate marker of programmed cell death.[52] We used this method for evaluating NaB-induced apoptosis in CA-MEK–expressing and –nonexpressing RIE cells. Consistent with our cell viability assay results, a high percentage of apoptotic cells was observed in the CA-MEK–nonexpressing cells (RIE-tiCAMEK cells with DOX and RIE-Mock cells), whereas a lower percentage were Annexin V positive in CA-MEK cells (RIE-tiCAMEK cells with vehicle and RIE-cCAMEK cells) (Figure 2*C*). Therefore, reduced cell viability seen in

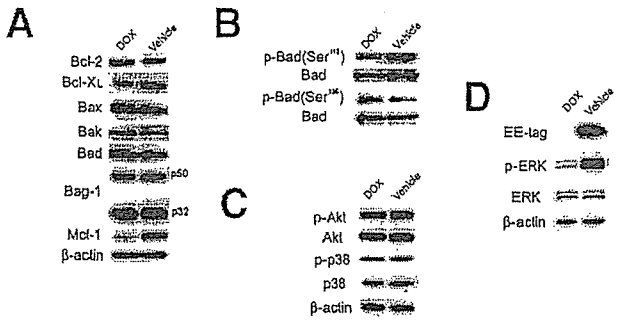

Figure 3. tiCAMEK expression modulates apoptosis-related proteins in RIE cells. (*A*) Western blot analysis of Bcl-2 family proteins in RIE-tiCAMEK cells with 2 μg/mL DOX (no CA-MEK expression) or vehicle (CA-MEK expression) at 72 hours following treatment with NaB. (*B*) Western blot analysis of in vitro phosphorylation of Bad. RIE-tiCAMEK cells were treated with 5 mmol/L NaB plus 2 μg/mL DOX (no CA-MEK expression) or vehicle (CA-MEK expression) for 72 hours. Anti-Bad indicates equal loading of recombinant Bad. (*C*) Western blot analysis of other kinase pathways in RIE-tiCAMEK cells. RIE-tiCAMEK cells were treated with 2 μg/mL DOX (no CA-MEK expression) or vehicle (CA-MEK expression) for 48 hours, and then the media was replaced with 5 mmol/L NaB plus 2 μg/mL DOX or vehicle for 24 hours following treatment with NaB. (*D*) Western blot analysis of EE-tagged CA-MEK, phospho-ERK1/2, and ERK1/2 protein levels in RIE-tiCAMEK cells under the same conditions as in *C*. β-actin indicates equal loading of protein in each sample.

Figure 2*A* and *B* is most likely due to increased programmed cell death.

MEK Activation Modulates Bcl-Family Homologues in NaB-Mediated Apoptosis

Because our data indicate a role for MEK in modulating resistance to NaB-induced apoptosis, we investigated the expression profile of the Bcl-2 family of proteins in RIE-tiCAMEK cells following treatment with NaB. Bcl-2 family members represent critical checkpoints in most apoptotic pathways, acting upstream of irreversible damage to cellular constituents.[53] CA-MEK expression (vehicle-treated cells) did not alter the expression levels of Bcl-2, Bax, Bad, and Bag-1 but did induce Bcl-X_L and Mcl-1 and reduced levels of Bak at 72 hours following treatment with NaB (Figure 3*A*). Phosphorylation of Bad at either Ser^{112} or Ser^{136} sites is believed to be required for inhibiting its proapoptotic function.[54] The phosphorylation of recombinant Bad at Ser^{112} was markedly increased in CA-MEK–expressing cells (vehicle-treated cells), whereas no difference at Ser^{136} was found (Figure 3*B*). These results are consistent with previous reports showing that there are at least 2 different pathways responsible for Bad phosphorylation; Ser^{112} is downstream of MAPK, whereas Ser^{136} is downstream of Akt.[55–58]

Other kinase pathways, such as c-Jun-N-terminal kinase (JNK),[59] p38,[3] and phosphatidylinositol 3-kinase/Akt,[60] can regulate programmed cell death. There was no evidence of crosstalk with the other kinase pathway, including the Akt and p38 pathways (Figure 3*C*). Moreover, Western blot analysis using 4 different antibodies failed to detect phosphorylated JNK (data not shown). However, along with increased levels of EE-tagged CA-MEK, elevated levels of phosphorylated-ERK1/2 signals were significantly increased only in CA-MEK–expressing cells (Figure 3*D*). Therefore, the antiapoptotic effect seen in CA-MEK–expressing cells is dependent on MEK-ERK signaling and not JNK, p38, or Akt.

MEK Activation Protects Against NaB-Induced Cell Cycle Arrest

Cell cycle progression is an important factor in oncogenic transformation. NaB is known to induce cell cycle arrest in intestinal epithelial cells. Cell cycle analysis of RIE-tiCAMEK cells with or without DOX at 72 hours following treatment of NaB was completed. Whereas RIE-tiCAMEK cells with DOX showed G_0/G_1 arrest, CA-MEK–expressing cells (vehicle) were resistant to NaB-mediated cell cycle arrest (Figure 4*A* and *B*). Elevated expression of cyclin D1 and cdk4 and decreased levels of $p27^{Kip}$ expression in the CA-MEK–expressing cells (vehicle) support this observation (Figure 4*C*). These results showed that CA-MEK signaling stimulated progression through the cell cycle. CA-MEK–expressing (vehicle-treated) cells did not alter the expression of cyclin E, cdk2, or p15 but did induce $p21^{Cip/WAF1}$ expression (Figure 4*C*; data not shown). Although $p21^{Cip/WAF1}$ was originally described as a universal inhibitor of cyclin-dependent kinases, recent studies have shown an increased expression of $p21^{Cip/WAF1}$ in some cancers. Therefore, the role of $p21^{Cip/WAF1}$ in cancer is being reevaluated.[61]

PG Production and COX-2 Levels in Cells Expressing CA-MEK

The presence of COX-2 and its derived PGs are known to provide cells with a distinct survival advantage.[34] Thus, we measured PG production and COX-2 expression in CA-MEK–expressing cells following NaB-mediated apoptosis. As shown in Figure 5*A*, CA-MEK–expressing cells (RIE-tiCAMEK with vehicle, RIE-cCAMEK) showed increased levels of 6-keto $PGF_{1\alpha}$ (a stable metabolite of PGI_2) at 48 hours following treatment with NaB. CA-MEK–expressing cells also produced increased levels of PGE_2 (Figure 5*B*). Along with increased PG production, elevated levels of COX-2 were observed in CA-MEK–expressing cells but not in RIE-tiCAMEK cells treated with DOX or in RIE-Mock cells (Figure 5*C*). Similar results were obtained using IEC-

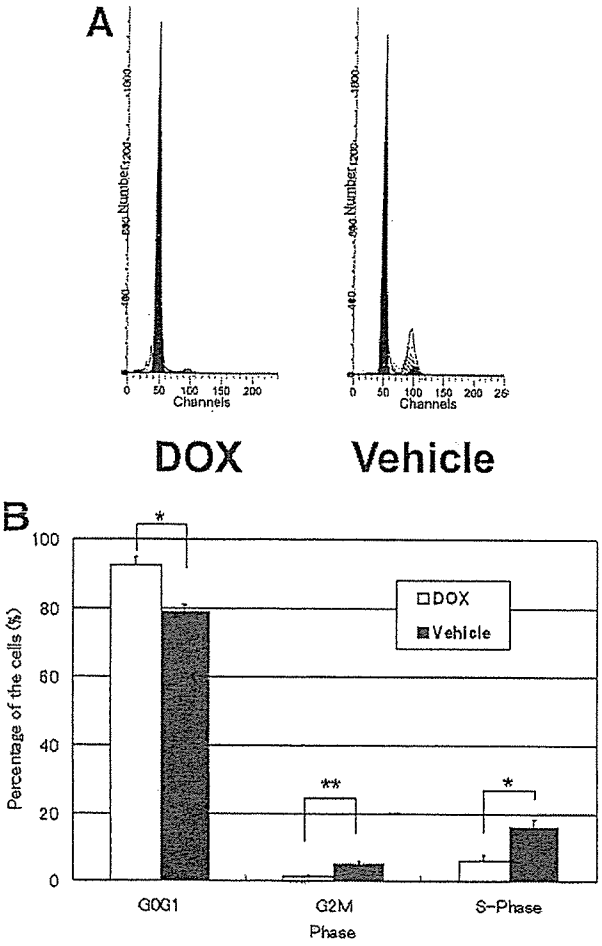

Figure 4. tiCAMEK-expressing cells are resistant to NaB-mediated cell cycle arrest. (*A*) Cell cycle analysis of RIE-tiCAMEK cells with DOX (no CA-MEK expression) or vehicle (CA-MEK expression) at 72 hours after treatment with NaB (5 mmol/L). (*B*) Results from *A* expressed as percentage of cells in each stage of the cell cycle. Values are the means ± SE of 3 separate experiments. $*P < .005$, $**P < .001$. (*C*) Western blot analysis of cyclin D1, cyclin E, cdk4, cdk2, p15, $p21^{Cip/WAF1}$, and $p27^{Kip}$ expression in RIE-tiCAMEK cells (with DOX or vehicle) following treatment with NaB for 72 hours.

cCAMEK and IEC-Mock cells (data not shown). COX-2 induction was confirmed by Northern blot analysis in RIE-tiCAMEK cells following the removal of DOX (Figure 5*D*). Therefore, increased levels of COX-2 accompanied by an increase in PGE_2 and PGI_2 may contribute to the antiapoptotic properties of CA-MEK.

To determine the involvement of ERK activation in the expression of COX-2, we established RIE-tiCAMEK cells with dominant negative ERK1 or 2 expression vectors. Western blot analysis shows that the overexpression of dominant negative ERK1 or 2 inhibits the expression of COX-2 in RIE-tiCAMEK cells (Figure 5*E*). This result confirms that ERK activity is involved in the CA-MEK–induced expression of COX-2.

Regulation of COX-2 in CA-MEK–Expressing Cells

To determine the mechanisms by which COX-2 expression is regulated in CA-MEK–expressing cells following treatment with NaB, we examined transcriptional and posttranscriptional regulation of the *COX-2* gene. Due to the internal expression of luciferase of RIE-tiCAMEK cells, we used RIE-cCAMEK and RIE-Mock cells for this study. A series of human *COX-2* promoter deletion constructs was transfected into RIE-cCAMEK and RIE-Mock cells. The transcriptional activity was increased in RIE-cCAMEK cells compared with RIE-Mock cells. The highest transcriptional activity was found with the full-length (−1432/+59) promoter construct (Figure 6*A*). Removal of the 2 nuclear factor κB sites, the upstream (−327/+59) and the downstream (−220/+59), or deletion of both the nuclear factor/interleukin-6 site (−124/+59) and the CRE (−52/+59) elements resulted in decreased transcriptional activity of COX-2. Mutating the CRE and/or nuclear factor/interleukin-6 site also reduced the transcriptional activity.

Posttranscriptional regulation via stabilization of COX-2 mRNA plays an important role in the regulation of COX-2 levels in a variety of cultured cells.[62] As shown in Figure 6*B* and *C*, COX-2 mRNA was rapidly degraded in RIE-tiCAMEK cells treated with DOX (half-life, <15 minutes). However, CA-MEK expression

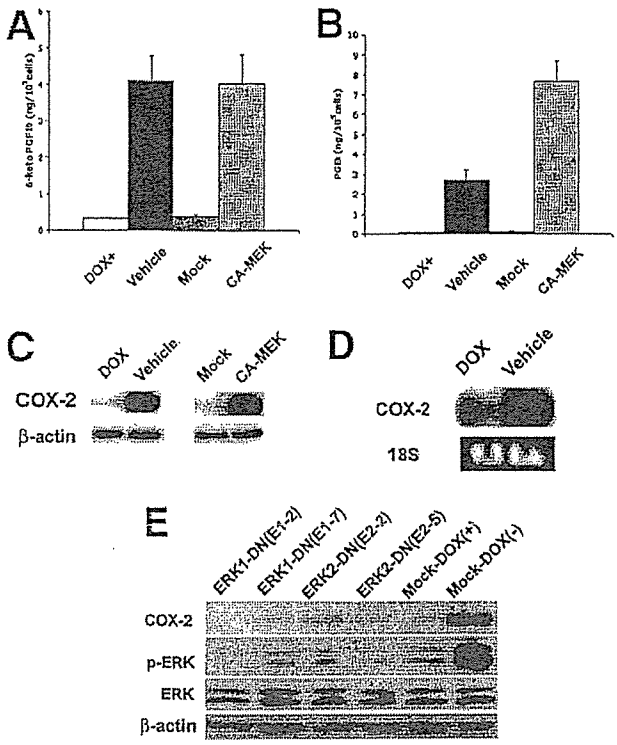

Figure 5. PG production and COX-2 expression following treatment with NaB. (*A*) 6-keto $PGF_{1\alpha}$ and (*B*) PGE_2 production at 48 hours after treatment with NaB in RIE-tiCAMEK cells with 2 μg/mL DOX (no CA-MEK expression) or vehicle (CA-MEK expression), RIE-Mock cells, and RIE-cCAMEK cells. Values are the means ± SE of 3 separate experiments performed in triplicate. (*C*) Western blot analysis of COX-2 at 48 hours following treatment with NaB in RIE-tiCAMEK cells (with 2 μg/mL DOX or vehicle), RIE-mock cells, and RIE-cCAMEK cells. (*D*) Northern blot analysis of COX-2 in tiCAMEK-expressing RIE cells with 2 μg/mL DOX or vehicle at 48 hours following treatment with NaB. (*E*) Sequential transfected RIE-tiCAMEK cells with ERK1 dominant negative vector or ERK2 dominant negative vector were established. Western blot analysis shows the expression levels of COX-2, phosphorylated ERK (p-ERK), ERK, and β-actin in the cells. ERK1 dominant negative clones (ERK1-DN; clone E1-2 and E1-7), ERK2 dominant negative clones (ERK2-DN; clone E2-2 and E2-5), and empty vector (pCEP4) transfected RIE-tiCAMEK cells (Mock) with or without DOX are shown.

markedly increased the stability of COX-2 mRNA and extended the half-life >60 minutes. These results indicated a marked increase in mRNA stability following MEK1 activation.

Pharmacologic Inhibition of COX-2 Inhibits MEK-Dependent Tumor Growth In Vivo by Stimulating Apoptosis

Overexpression of COX-2 frequently occurs in a variety of human malignancies, including those of colon, lung, breast, skin, and esophagus.[32] We have shown that COX-2 is strongly expressed in MEK-transformed cells and may induce antiapoptotic properties. To verify this hypothesis in vivo, we treated mice xenografted with IEC-cCAMEK cells and RIE-cCAMEK cells with a COX-2–selective inhibitor (celecoxib) and evaluated the growth rate of MEK-induced tumors. As shown in Figure 7*A*, treatment of IEC-cCAMEK xenografted mice with celecoxib for 17 days induced a 72% reduction in tumor volume when compared with vehicle-treated controls. A similar inhibitory effect was also found with celecoxib-treated RIE-cCAMEK (clone DD14) tumors, although to a lesser extent (Figure 7*A*). Western blot analysis of tissues from the excised tumors showed that the expression of Bcl-X_L was decreased in the celecoxib-treated mice (Figure 7*B*). Moreover, increased apoptotic staining was seen in the celecoxib-treated tissues. We observed 0.7 ± 0.3 apoptotic IECcCAMEK(D1) cells per field in untreated mice (vehicle) and 5.9 ± 2.1 apoptotic IECcCAMEK(D1) cells per field in celecoxib-treated mice (Figure 7*C*). Similarly, we found 1.2 ± 0.5 apoptotic RIEcCAMEK(DD14) cells per field in untreated mice (vehicle) and 5.1 ± 1.7 apoptotic RIEcCAMEK(DD14) cells per field in celecoxib-treated mice (Figure 7*C*). Therefore, the in vivo effects of celecoxib on tumor volume may also occur through the inhibition of the antiapoptotic properties of COX-2, including the down-regulation of Bcl-X_L.

Discussion

Here we show that CA-MEK1 transforms both RIE-1 and IEC-6 rat intestinal epithelial cells. This finding is in agreement with a recent report showing the ability of CA-MEK to transform IEC-6 cells.[24] However, Oldham et al[23] reported that constitutively active mutants of Raf-1 did not transform RIE-1 and IEC-6 cells. This finding shows key differences between the Raf-1 and CA-MEK signaling cascades in intestinal epithelial cells. Recent evidence shows that Raf-1 may use multiple effectors other than MEK-ERK to mediate its cellular effects. For example, activated Raf-1, but not MEK, can drive the differentiation of hippocampal neuronal cells, whereas mutant Raf-1, defective in MEK activation, is still capable of activating selected signaling pathways.[63,64] Although Raf-1 activates MEK1, these observations suggest a significant difference between downstream signals affected by the activation of Raf-1 and MEK1 pathways.

In this study, we show that MEK-ERK signaling can protect against NaB-mediated apoptosis. Several reports provide evidence that the MEK-ERK pathway has been implicated in protection from various proapoptotic signals via modulation of antiapoptotic proteins such as Bcl-X_L, Bcl-2, and Mcl-1.[10–14] Our results suggest that the CA-MEK signal up-regulates the expression of Bcl-

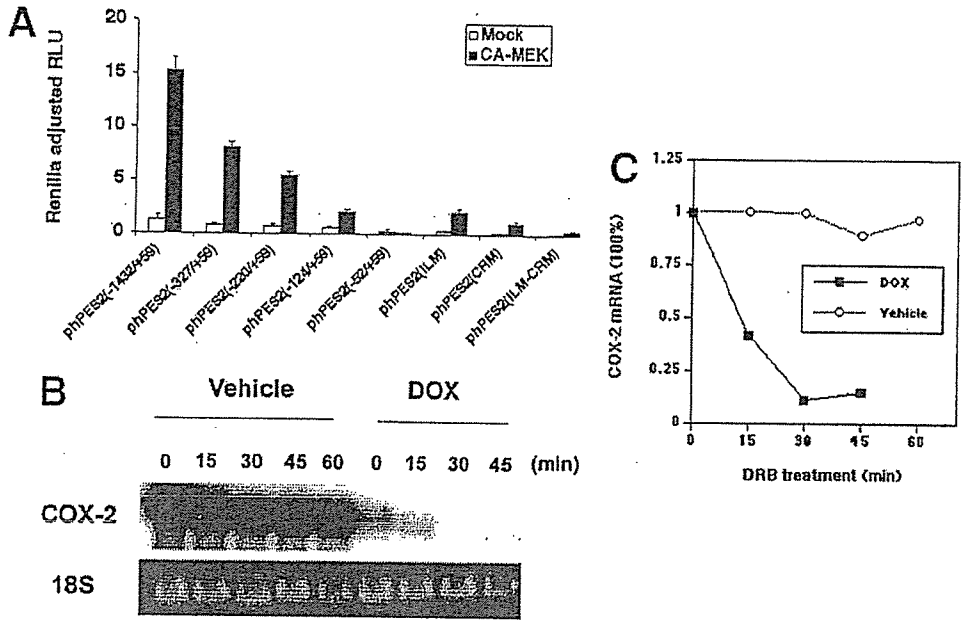

Figure 6. COX-2 regulation in CA-MEK cells following treatment with NaB. (*A*) COX-2 promoter assay using cCAMEK-expressing RIE cells compared with the mock-transfected cells at 24 hours after treatment with NaB are shown. The values are shown as *Renilla* adjusted luciferase values and represent the average ± SD of 3 independent experiments performed in quadruplicate. (*B*) Northern blot analysis of COX-2 mRNA in tiCAMEK-expressing RIE cells with and without DOX. RIE-tiCAMEK cells with DOX or vehicle were treated with 5 mmol/L NaB for 24 hours. Further transcriptional activity was blocked by the addition of 100 μmol/L of 5,6-dichlorobenzimidazole riboside. Total RNA samples were isolated at the indicated times and probed with a labeled COX-2 complementary DNA probe. (*C*) The results from *B* were analyzed by densitometry scanning using the Scion Image program and normalized by 18S.

X_L, Mcl-1, and COX-2. Previously, we have shown that forced expression of COX-2 leads to the inhibition of apoptosis in intestinal epithelial cells.[34] Therefore, COX-2 may play an important role in MEK-associated resistance to apoptosis. We observed an increased rate of apoptosis and decreased levels of Bcl-X_L in the MEK-induced tumors. Other groups have reported that the treatment of APC^{min} mice with a COX-2–selective inhibitor resulted in increased apoptosis and a reduction of Bcl-X_L levels in adenomatous polyps.[65] Additionally, Mcl-1 is also regulated via COX-2 signaling. Mcl-1 levels are tightly regulated by COX-2 in human lung adenocarcinoma cells and contribute to cell survival.[66] Therefore, the regulation of these antiapoptotic proteins may be linked to the expression of COX-2. Conversely, phosphorylation of Bad on Ser^{112} or Ser^{136} dissociates Bcl-X_L/Bad heterodimers and unmasks the antiapoptotic effect of Bcl-X_L.[54,58] Therefore, increased phosphorylation of Bad (Ser^{112}) may also act as a negative regulator of apoptosis in CA-MEK–expressing cells. Collectively, elevated expression of antiapoptotic Bcl-X_L, Mcl-1, and COX-2, reduced expression of the proapoptotic protein Bak, and inhibition of the proapoptotic effect of Bad may all contribute to the antiapoptotic effects of CA-MEK–expressing cells.

Cell cycle progression is another important aspect of tumorigenesis. Abnormalities in the expression of cell-cycle regulatory proteins have been reported in tumors of the small bowel and in colorectal carcinomas.[67] We show here that MEK activation leads to elevated levels of cyclin D1 and cdk4 and decreased levels of $p27^{Kip}$ expression that may confer resistance to NaB-mediated cell cycle arrest. Furthermore, we did not observe any changes in cyclin E or cdk2 expression. Similar findings were published by Boucher et al,[24] who showed that cyclin D1, cdk2, and cdk4 proteins were increased and $p27^{Kip}$ was decreased in CA-MEK–expressing IEC-6 cells. Additionally, another report shows that the activation of the MAPK cascade was required for S-phase entry and $p27^{Kip}$ down-regulation in IEC-6 cells.[68] Therefore, the activation of the MEK pathway may contribute to cell G_0/G_1 progression via cyclin D1 and cdk4 as well as S-phase entry by down-regulating $p27^{Kip}$, at least in rodent immortalized intestinal cells. In contrast to the forced mitogenesis of CA-MEK–expressing IEC-6 cells, Boucher et al[24] also showed CA-MEK–induced cell cycle arrest in nonimmortalized human intestinal epithelial cells by up-regulating $p21^{Cip/WAF1}$, p53, and p16 expression. They suggested that the noninduction of $p21^{Cip/WAF1}$, p53, and p16 in CA-MEK–expressing

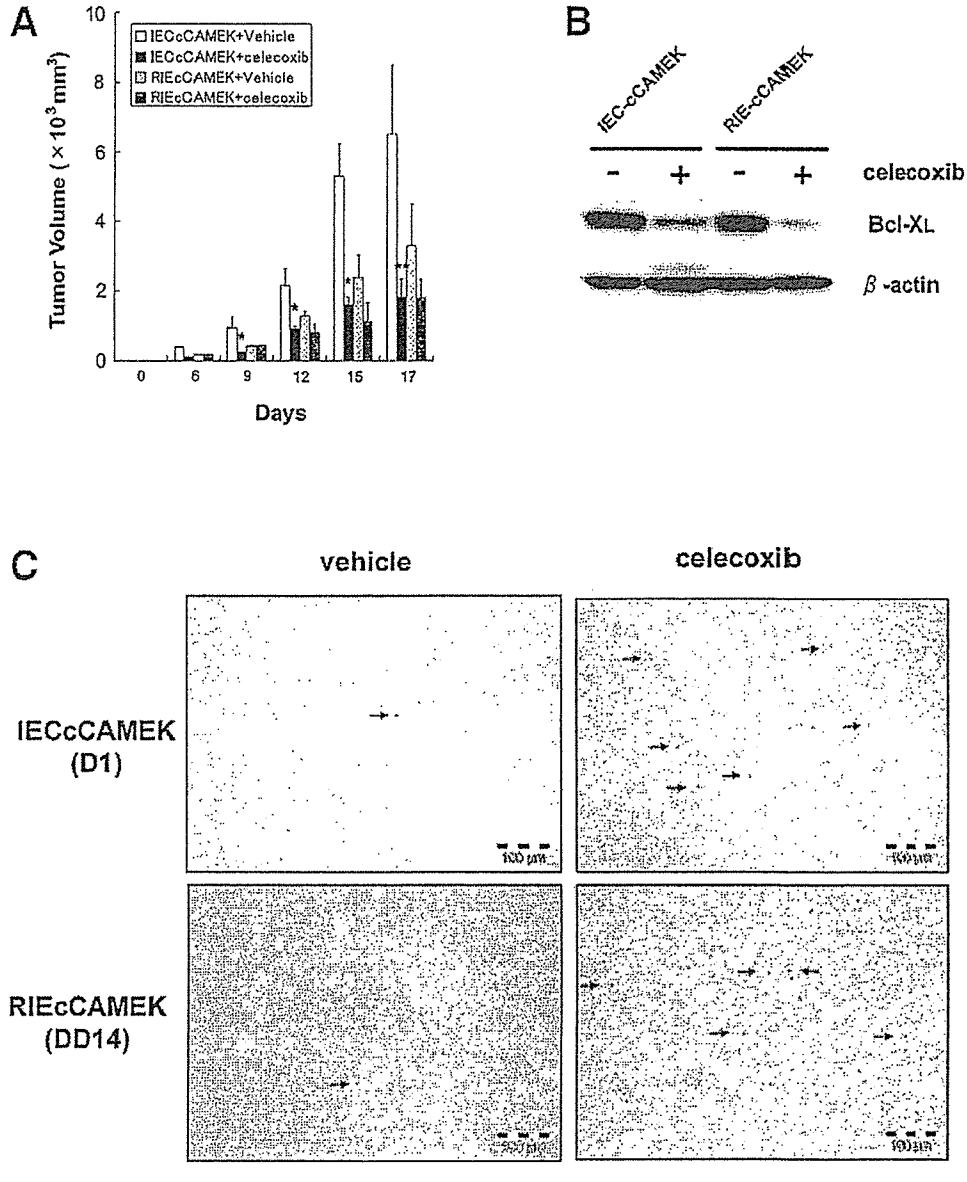

Figure 7. Pharmacologic inhibition of COX-2 reduces cCAMEK-expressing tumor growth in vivo. (*A*) A total of 1×10^6 IEC-cCAMEK (clone D1) and RIE-cCAMEK (clone DD14) cells suspended in 0.2 mL DMEM were injected into the dorsal subcutaneous tissue of athymic nude mice. Mice were given a COX-2–selective inhibitor (celecoxib, 100 mg/kg) (n = 3 mice per each group; n = 3 mice in the RIE-cCAMEK injected group) or vehicle (n = 3 mice per each group) by daily gavage, and the treatment was continued for 17 days. Tumor volumes were calculated from measurements taken at the indicated times and calculated using the following formula: $V = L \times W^2 \times 0.5$, where *V* is volume, *L* is length, and *W* is width. Data are represented as the average of each group ± SD. $*P < .05$, $**P < .01$ vs vehicle-treated control. (*B*) Western blot analysis of tissues from IEC-cCAMEK (clone D1) and RIE-cCAMEK (clone DD14) tumors. β-actin indicates equal protein loaded in each lane. (*C*) Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling staining of IEC-cCAMEK (D1) and RIE-cCAMEK (DD14) tissue sections. *Arrows* indicate brown-stained apoptotic cells.

IEC-6 cells may contribute to these differences. However, we find that $p21^{Cip/WAF1}$, a universal inhibitor of cyclin-dependent kinases, was increased in CA-MEK–transformed cells. Here, we show that CA-MEK–expressing RIE cells were resistant to NaB-mediated cell cycle arrest and apoptosis despite the up-regulation of $p21^{Cip/WAF1}$. $p21^{Cip/WAF1}$ is also reported to act as an adaptor protein to promote assembly of the active cdk4/cyclinD1 complex,[69] and the forced expression of cyclin D1 in human glioma and rodent fibroblast cells induced $p21^{Cip/WAF1}$ expression without altering cell cycle progression.[70] Moreover, recent studies have shown that $p21^{Cip/WAF1}$ can act as an antiapoptotic and growth-promoting protein in addition to its known growth inhibitory role.[71–73] The overexpression of $p21^{Cip/WAF1}$ is an early event in the development of some neoplasms, and $p21^{Cip/WAF1}$ is currently being evaluated as a therapeutic cancer target.[61,74] Therefore, we speculate that the increase of $p21^{Cip/WAF1}$ following MEK activation may have an antiapoptotic and growth-promoting role, at least in this context.

We have clearly shown that the activation of MEK signaling alone is sufficient to induce COX-2 in rat intestinal epithelial cells at both transcriptional and posttranscriptional levels. Therefore, COX-2–derived bioactive lipids may play a key role in tumor formation via activating the MEK-ERK cascade. Surprisingly, CA-MEK–expressing cells produce not only PGI_2 but also PGE_2. These cells do not normally produce PGE_2,[45] but PGE_2 levels are increased in human colon cancer tissue

compared with surrounding normal mucosa.[75] PGE_2 has also been reported to inhibit programmed cell death and enhance invasiveness of colorectal carcinoma cells.[76] Furthermore, PGE_2 can activate epidermal growth factor receptor,[77] which leads to ERK activation and can trigger ERK2-mitogenic signaling in gastric epithelial and colon carcinoma cells.[78]

We show that the expression of CA-MEK results in the transformation of rat intestinal epithelial cells and that activation of the MEK-ERK cascade suppresses programmed cell death and leads to tumorigenesis. COX-2 may also play an important role in MEK-induced tumor formation through stimulation of resistance to apoptosis. These results also indicate that the MEK-ERK signaling pathway may provide an important target for developing new cancer drugs. We suspect that MEK-ERK kinase inhibitors are currently being developed for clinical use.

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Contribution of catechol *O*-methyltransferase to the removal of accumulated interstitial catecholamines evoked by myocardial ischemia

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Abstract

Catechol *O*-methyltransferase (COMT) plays an important role for clearance of high catecholamine levels. Although myocardial ischemia evokes similar excessive catecholamine accumulation, it is uncertain whether COMT activity is involved in the removal of accumulated catecholamines evoked by myocardial ischemia. We examined how COMT activity affects myocardial catecholamine levels during myocardial ischemia and reperfusion. We implanted a dialysis probe into the left ventricular myocardial free wall and measured dialysate catecholamines levels in anesthetized rabbits. Dialysate catecholamine levels served as an index of myocardial interstitial catecholamine levels. We introduced myocardial ischemia by 60 min occlusion of the main coronary artery. The ischemia-induced dialysate catecholamines levels were compared with and without the pretreatment with entacapone (COMT inhibitor, 10 mg/kg, i.p.). Acute myocardial ischemia progressively increased dialysate catecholamine levels. Acute myocardial ischemia increased dialysate norepinephrine (NE) levels (20,453 ± 7186 pg/ml), epinephrine (EPI) levels (1724 ± 706 pg/ml), and dopamine (DA) levels (1807 ± 800 pg/ml) at the last 15 min of coronary occlusion. Inhibition of COMT activity by entacapone augmented the ischemia-induced NE levels (54,306 ± 6618 pg/ml), EPI levels (2681 ± 567 pg/ml), and DA (3551 ± 710 pg/ml) levels at the last 15 min of coronary occlusion. Myocardial ischemia evoked NE, EPI, and DA accumulation in the myocardial interstitial space. The inhibition of COMT activity augmented these increments in NE, EPI, and DA. These data suggest that cardiac COMT activity influences on the removal of accumulated catecholamine during myocardial ischemia.

Keywords: Catecholamine; Microdialysis; Myocardial infarction; Heart; Catechol *O*-methyltransferase

Myocardial ischemia evokes an excessive norepinephrine (NE) accumulation in the myocardial interstitial space [2,15]. Interstitial NE is largely removed by NE transport into the sympathetic nerve endings and metabolized to dihydroxyphenylglycol (DHPG) via monoamine oxidase (MAO) [6,22]. The remainder spills over into the coronary sinus [6]. However, during myocardial ischemia, two important NE removing systems are impaired. Myocardial ischemia reduces coronary flow, which abolishes NE spillover. Furthermore, membrane NE transport is dependent on the Na^+ gradient between the extracellular and intracellular spaces. During ischemia, NE uptake is blocked and outward NE transport through the uptake$_1$ carrier is induced by the reduced Na^+ gradient [17]. Thus, production of DHPG via MAO is inhibited by myocardial ischemia [1]. Up to now, little has been known about the role of catechol *O*-methyltransferase (COMT) in the removal of interstitial NE. Catechol *O*-methyltransferase has been believed to be operative only at high concentrations of NE via NE infusion [12]. An excessive NE accumulation in the myocardial ischemia was similar to NE levels in

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intravenous NE infusion. Therefore, this NE removal system may be the sole mechanism that decreases myocardial interstitial NE.

Recent study has demonstrated that myocardial ischemia is associated with a pronounced increase in the concentration of endogenous NE, epinephrine (EPI), dopamine (DA) in the myocardial interstitial space [14]. These accumulated catecholamines may be a candidate of substrate of COMT. However, in most experiments on COMT activity, isoprenaline was used as the substrate of COMT [11,19] since it is not a substrate for neuronal uptake and MAO activity. Furthermore, data on isolated perfused lungs suggest that the affinity of COMT activity for *O*-methylation differed among the three amines [3]. It is uncertain whether COMT activity is involved in the removal of accumulated interstitial catecholamines evoked by myocardial ischemia.

In the present study, the possibility that the concentration of these three catecholamines in the myocardial interstitial space was affected by COMT activity was examined in anesthetized myocardial ischemic rabbits. With the use of dialysis technique, a dialysis probe was implanted into the left ventricle free wall perfused by the main branch of left circumflex coronary artery (LCX) to measure myocardial interstitial catecholamines levels in the ischemic region and dialysate catecholamines levels were compared in the absence and presence of COMT inhibitor.

Animal care proceeded in strict accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult male Japanese white rabbits (2.5–3.2 kg) were anesthetized with pentobarbital sodium (30–35 mg/kg i.v.). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg/kg/h). The rabbits were intubated and ventilated with room air mixed with oxygen. Heart rate, arterial pressure, and electrocardiogram were simultaneously monitored with a data recorder. The fifth or sixth rib on the left side was partially removed to expose the heart. A 4–0 silk suture was passed around the main branch of LCX, to act as the occluder for later coronary occlusion. With a fine guiding needle, a dialysis probe was implanted in the region perfused by LCX of the left ventricular wall. Judging from changes in the color of the ventricular wall during a brief coronary occlusion, the dialysis probe was located in the midst of the ischemic region. Heparin sodium (100 IU/Kg) was administered intravenously to prevent blood coagulation.

The dialysate NE, EPI, and DA levels were measured as an index of myocardial interstitial NE, EPI, and DA levels, respectively. A dialysis fiber (8 mm length, 0.31 mm o.d., and 0.20 mm i.d.; PAN-1200 50,000 molecular weight cutoff, Asahi Chemical Japan) was glued at both ends of a polyethylene tube. The dialysis probe was perfused with Ringer's solution at a perfusion speed of 2 μl/min. Dialysate NE level was measured by the first HPLC after removing interfering compounds by the alumina procedure [23]. Dialysate EPI and DA levels were measured by direct injection into the second HPLC [18].

After control sampling, we occluded the main branch of LCX for 60 min and then released the occluder. The 15-min dialysate samples were collected before, during and after 60 min LCX occlusion. In vehicle group, we administered saline intraperitoneally as vehicle 120 min before control sampling. After control sampling, we observed the time course of dialysate NE, EPI, and DA levels from the ischemic region during 60 min of coronary occlusion and 15 min of reperfusion. To elucidate the role of COMT activity in the ischemia-induced changes in myocardial interstitial NE EPI, and DA levels, we compared dialysate NE, EPI, and DA levels in the ischemic region with those levels after injection of COMT inhibitor. We administered intraperitoneally the COMT inhibitor entacapone (10 mg/kg; Orion-Pharma, Espoo, Finland) 120 min before control sampling. Entacapone was dissolved in phosphate buffered saline, the pH of the solution was adjusted to 7.4, and the dose of entacapone was determined based on the dose used in the earlier preliminary experiments [7,8].

Changes in the dialysate NE levels in the vehicle and the pretreatment with entacapone are shown in Fig. 1. In the vehicle group, dialysate NE level averaged from six rabbits was 52 ± 12 pg/ml in the control. During 60 min coronary occlusion, dialysate NE levels markedly increased. The dialysate NE levels reached up to 400 times the control levels during the last 15 min of 60 min coronary occlusion. After release of the occluder, dialysate NE levels rapidly decreased to 3473 ± 735 pg/ml, although their levels were higher than those in the control. In the presence of entacapone, dialysate NE levels also markedly increased during 60 min coronary occlusion. The dialysate NE levels reached up to 1000 times the control levels during the last 15 min of 60 min coronary occlusion. These increases in dialysate NE levels at 15–60 min of coronary occlusion were significantly enhanced by entacapone whereas entacapone did not change dialysate NE levels in the control (51 ± 16 pg/ml) or at 0–15 min of

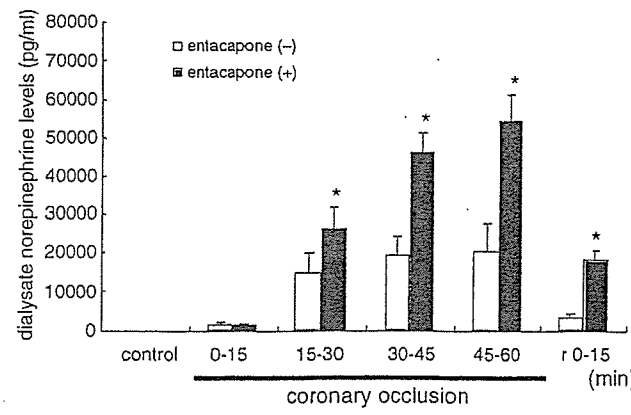

Fig. 1. Dialysate norepinephrine levels before, during and after 60 min-coronary occlusion. Values are mean ± S.E. ($n = 6$). $^*P < 0.05$ vs. concurrent value of vehicle group.

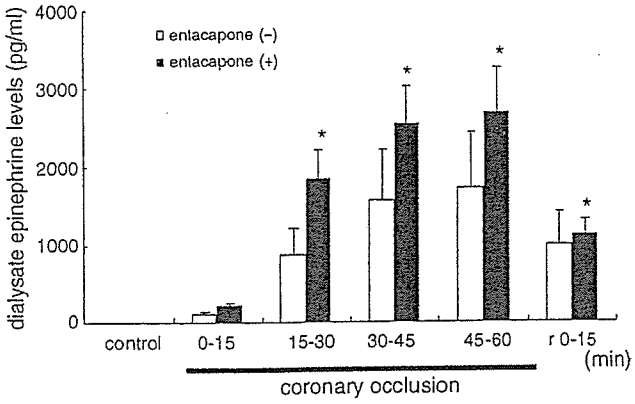

Fig. 2. Dialysate epinephrine levels before, during and after 60 min-coronary occlusion. Values are mean ± S.E. ($n = 6$). $^*P < 0.05$ vs. concurrent value of vehicle group.

coronary occlusion. Dialysate NE levels decreased by reperfusion, but remained higher than those in vehicle group. Thus, COMT activity for NE removal was operative in ischemic and reperfusion periods. Entacapone augmented peak NE levels to 160% of vehicle group.

Dialysate EPI levels were below the detectable level in the control. After coronary occlusion, dialysate EPI levels gradually increased and reached 1724 ± 706 pg/ml at 45–60 min of occlusion (Fig. 2). Peak EPI levels during the ischemia were one-twentieth of NE levels during the ischemic period. In the presence of entacapone, dialysate EPI levels were below the detectable level in the control. Dialysate EPI levels gradually increased during coronary occlusion and reached 2681 ± 567 pg/ml at 45–60 min of occlusion. In the presence of entacapone, dialysate EPI levels at 15–60 min of the ischemia were higher than those in the vehicle group. Entacapone augmented peak EPI levels by 50% of vehicle group.

Dialysate DA levels were below the detectable level in the control and at 0–15 min of ischemia. After 15 min of occlusion, dialysate DA levels gradually increased and reached 1807 ± 800 pg/ml at 45–60 min of occlusion (Fig. 3). Peak DA levels during the ischemia were one-twentieth of NE

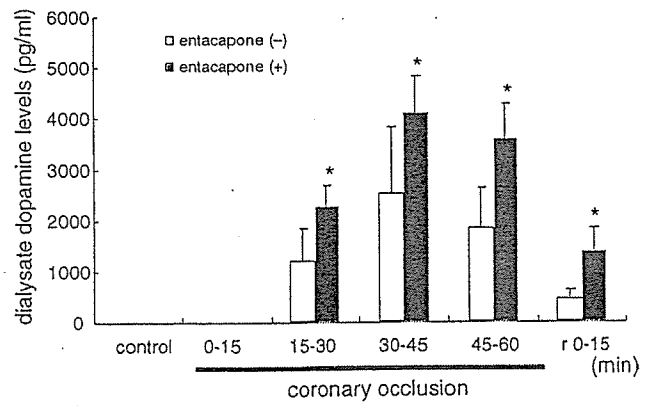

Fig. 3. Dialysate dopamine levels before, during and after 60 min-coronary occlusion. Values are mean ± S.E. ($n = 6$). $^*P < 0.05$ vs. concurrent value of vehicle group.

levels during the ischemia. In the presence of entacapone, dialysate DA levels were below the detectable level in the control and at 0–15 min of the occlusion. Dialysate DA levels gradually increased during 15–60 min of the ischemia and reached 3351 ± 710 pg/ml at 45–60 min of occlusion. In the presence of entacapone, dialysate DA levels at 15–60 min of the ischemia were higher than those in the vehicle group. Entacapone augmented peak DA levels by 100% of vehicle group.

Myocardial ischemia induced a progressive increase of interstitial catecholamines. The rank order of the amount of catecholamine release was NE much greater than EPI or DA, with this rank order remaining unchanged before, during and after myocardial ischemia. These findings are in line with those reported by Lameris et al. [14] studied the time course of myocardial interstitial catecholamine levels during myocardial ischemia. The measurement of overall content in the left ventricle free wall was performed in dogs [13] and the in vitro ratio of EPI/NE or DA/NE was similar to our result. Therefore, the rank order may reflect the ratio of overall catecholamine content in the left ventricle free wall.

In the resting state and early period (0–15 min) of ischemia, COMT does not appear to contribute to the removal of myocardial interstitial catecholamine levels. In the mid-late period of ischemia, COMT contributes to the inactivation of high myocardial interstitial catecholamine levels evoked by ischemia. The rank order of the amount of neurotransmitter release was NE much greater than EPI or DA. From percentage increase of catecholamine by entacapone, the rank order of COMT activity for removal of catecholamines was considered to be NE greater than DA greater than EPI. On the other hand, when catecholamines were infused in the isolated rat heart, the metabolism of the catecholamines by COMT differs: DA = NE less than EPI [9]. These data suggest that contribution of COMT to removal of accumulated catecholamines depends on the types of amines and the amount of accumulated catecholamine. In the absence of catecholamine spill over and MAO activity, COMT might constitutes one of major pathways of catecholamine metabolism in ischemic heart. Alternatively all three catecholamines are taken up and then metabolized by COMT at the extraneuronal tissues [4,10]. Uptake and *O*-methylation may handle three catecholamines in a different manner.

Up to now, little has been known about the role of cardiac COMT activity in the removal of accumulated interstitial catecholamine. In the isolated perfused rat, Carlsson et al. [5], demonstrated that marked NE release was paralleled by an increasing extraneuronal inactivation of released NE. Accumulated catecholamine in myocardial interstitial space is involved in the pathophysiology of ischemic heart disease [16,21]. Therefore, these data suggest that inhibition of COMT activity deteriorates myocardial ischemic injury via enhanced catecholamine accumulation. In contrast to this hypothesis, Valenza et al. [20], demonstrated that the inhibition of COMT (by nitecapone) improved the mechanical function of the heart during ischemia-reperfusion injury. In

the present study, we did not measure myocardial contractile function or biochemical markers. Future work should concentrate on these aspects of COMT action during myocardial ischemia.

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Hypertonic sodium chloride induction of cyclooxygenase-2 occurs independently of NF-κB and is inhibited by the glucocorticoid receptor in A549 cells

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Abstract Cellular response to a hypertonic environment is important for fluid clearance in the lung. Hypertonicity modulates prostaglandin synthesis by influencing cyclooxygenase-2 (COX-2) expression in tissues such as liver and kidney via a mitogen-activated protein kinase (MAPK)-dependent pathway. However, little is known about COX-2 expression in response to hypertonicity in the lung. COX-2 mRNA accumulation induced by hypertonic NaCl was detected after 1 h of treatment, and COX-2 mRNA continued to accumulate until 18 h, the longest time point examined, in human alveolar epithelial A549 cells. This induction was a transcriptional event that occurred in the absence of the protein synthesis inhibitor cycloheximide and was the result of enhanced promoter activity, as examined with the use of full-length COX-2 promoter-driven reporter plasmids. The induction of COX-2 expression by hypertonic NaCl did not require the activation of NF-κB. The p38 MAPK inhibitor, SB203580, or MEK1/2 inhibitor, U0126, inhibited hypertonic induction of COX-2 expression. We examined whether the hypertonic induction of COX-2 was under the influence of glucocorticoid; we found that COX-2 promoter activity and mRNA and protein levels were depressed by dexamethasone and antagonized by the glucocorticoid receptor (GR) antagonist RU486. Our data demonstrate that the induction of COX-2 expression by hypertonic NaCl occurs independently of NF-κB and is inhibited by the GR in A549 cells.

Keywords: COX-2; Hypertonic sodium chloride; NF-κB; Glucocorticoid receptor; Alveolar epithelial cells

1. Introduction

Salt and water transport play an important role in alveolar fluid clearance (AFC), and active sodium ion transport drives osmotic water transport in the lung. The alveoli must remain open and free from fluid for efficient gas exchange to occur [1]. Intact AFC, therefore, is critical in clearing fluid from the lungs at birth and keeping the alveolar space relatively fluid-free for adequate gas exchange under physiological conditions [2–4].

One enzyme that may be involved in this process is cyclooxygenase (COX), a key regulatory enzyme in the biosynthesis of prostaglandins (PGs) from arachidonic acid [5]. COX-2 has a diverse assortment of biological functions in mammalian tissues, such as regulation of vascular tone, expression and secretion of rennin, and salt and water homeostasis in the kidneys [6]. Recent studies on the effects of salt on COX-2 expression in the kidney have identified some mechanisms for the regulation of COX-2 expression. It was reported that a low-salt medium regulates COX-2 expression by p38- and NF-κB-dependent signaling pathways in cultured cortical cells from the thick ascending limb of the loop of Henle [6,7]. Hypertonic NaCl activated COX-2 in renal medullary interstitial cells through the transactivation of the epidermal growth factor receptor [8]. Pathways involving the transcription factor NF-κB and mitogen-activated protein kinase (MAPK) play a central role in the high-salt-mediated regulation of COX-2 expression in mammalian kidney cells [9,10]. All three members of the MAPK family (ERK, JNK-2, and p38) as well as Src kinases are required for tonicity-stimulated COX-2 expression in inner medullary collecting duct cells [10].

Cellular dysfunction induced by hypertonic NaCl in alveolar epithelial cells could play an important role in the lung fluid balance under both normal and pathological conditions. Saline infusion has been reported to have a significant influence on inflammation-related gene expression in the lung [11]. Previous studies have shown that the inhibition of prostaglandin synthesis inhibits the flow of liquid from the fetal lungs [12–14]. However, the effects of hypertonic stress on COX-2 expression in alveolar epithelial cells and the mechanism involved are not known. In the present study, we examined the hypertonic NaCl induction of COX-2 expression in the human alveolar epithelial cell line A549.

2. Materials and methods

2.1. Materials

Cycloheximide, pyrrolidine dithiocarbamate (PDTC), SB203580, U0126, urea, NaCl, mannitol, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1), aldosterone, spironolactone, dexamethasone, RU486, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum (FCS), Trizol Reagent, and penicillin/streptomycin were purchased from GIBCO Invitrogen (Grand Island, NY, USA).

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2.2. Cell culture, transfection, and luciferase assays

A549 cells, a human pulmonary epithelial cell line, were grown in DMEM/Ham's F12 nutrient mixture containing 10% FCS and penicillin/streptomycin in a humidified 37 °C incubator. COX-2-Luc, a firefly luciferase reporter construct containing the mouse COX-2 gene promoter fragment (3.4 kb), −327/+59, a firefly luciferase reporter deletion construct of the human COX-2 promoter, KBM, an NF-κB binding region site-specific mutant of −327/+59, hα ENaC-Luc, a firefly luciferase reporter construct containing the human α ENaC promoter fragment (1.4 kb), and 3 × (NF-κB)tk-Luc, a firefly luciferase reporter construct containing three repeated NF-κB-responsive elements, were kindly provided Dr. Huifang Cheng (Vanderbilt University School of Medicine, Tennessee, USA) [6], Dr. Hiroyasu Inoue (Nara Women's University, Nara, Japan) [15], Dr. Christie P. Thomas (University of Iowa College of Medicine and the Veterans Affairs Medical Center, Iowa, USA) [16], and Dr. Sam Okret (Karolinska University Hospital Huddinge, Huddinge, Sweden) [17], respectively. A549 cells were transiently transfected with COX-2-Luc, −327/+59, KBM, hα ENaC-Luc, or 3 × (NF-κB)tk-Luc by electroporation. Electroporation was performed with a Gene Pulser II (Bio-Rad, Hercules, CA, USA). Cells were trypsinized, washed in cold PBS, and resuspended in PBS. A 400 μl portion of the suspension was mixed with 20 μg of plasmid DNA.

After 5 min at room temperature, cells were pulsed at 1000 μF and 250 V. After 10 min incubation at 37 °C, the suspension was diluted in medium and cultured for 24 h. Cells were replaced with fresh medium and treated with high salt (100 mM NaCl; 200 mosmol/kgH_2O) to the normal medium resulting in final osmolarity of 500 mosmol/kgH_2O or pretreated with specific inhibitors for 30 min before treatment of high salt for 18 h. After treatment, the cells were harvested and lysed with reporter lysis buffer (Promega Luciferase Assay system). The cell extract was mixed with the luciferase assay reagent and analyzed by the luminometer (Lumat LB 9507, EG&G Berthold, Bad Widbad, Germany).

2.3. Reverse transcription-PCR

Total RNA was extracted using Trizol Reagent according to the manufacturer's instruction. RNA pellets were dissolved in diethylpyrocarbonate-treated water. The yield of RNA was quantified by spectroscopy at 260 nm. Samples were aliquoted and stored at −80 °C until further processing. To synthesize first strand cDNA, 3 μg total RNA was incubated at 70 °C for 5 min with 0.5 μg of random hexamer and deionized water (up to 11 μl). The reverse transcription (RT) reaction was performed using 40 U of M-MLV reverse transcriptase (Promega, Madison, WI, USA) in 5 × reaction buffer (250 mmol/l Tris–HCl; pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), RNase inhibitor at 1 U/μl, and 1 mM dNTP mixtures at 37 °C for 60 min. The reaction was terminated by heating at 70 °C for 10 min, followed by cooling at 4 °C. The resulting cDNA was added to the PCR mixture containing 10 × PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 25 U of rTaq polymerase (TakaRa, Shiga, Japan), 4 μl of 2.5 mM dNTP mixtures, and 10 pmol of primers each. The final volume was 50 μl. Samples were amplified at 94 °C for 5 min, 23 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s using Mastercycler gradient (Eppendorf, Hamburg, Germany). β-actin was amplified for 20 cycles, followed by 72 °C for 5 min. The primers used were: COX-2 sense primer, 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′; COX-2 antisense primer, 5′-AGATCATCTCTGCCTGAGTATCTTT-3′[18]; β-actin sense primer, 5′-CCTGACCCTGAAGTACCCCA-3′, β-actin antisense primer, 5′-CGTCATGCAGCTCATAGCTC-3′; IL-8 sense primer, 5′-AAGGAACCATCTCACTG-3′, IL-8 antisense primer, 5′-GATTCTTGGATACCACAGAG-3′. The expected size of amplicons for COX-2, ®-actin, and IL-8 are 305, 550, 500, and 369 bp, respectively.

2.4. Western blot analysis

Protein extracted from A549 cells was isolated in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS) with protease inhibitor cocktail (Sigma) on ice for 1 h and then centrifuged for 20 min at 13000 × g. Supernatant was collected and protein concentrations were measured using the Bradford method (Bio-Rad). Proteins were dissolved in sample buffer and boiled for 5 min prior to loading onto an acrylamide gel. After SDS–PAGE, proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 60 min at room temperature. The membranes were incubated for 2 h at room temperature with 1:1000 dilution of COX-2 polyclonal antibody (Cayman, Ann Arbor, MI, USA). Equal lane loading was assessed using β-actin monoclonal antibody (Sigma). After washing with TBST, blots were incubated with 1:5000 dilution of the horseradish peroxidase conjugated-secondary antibody (Zymed, San Francisco, CA, USA), and washed again three times with TBST. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.5. Statistical analysis

Data were expressed as means ± S.E.M., and statistical analysis for single comparison was performed using the Student's t test. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Hypertonic NaCl induces COX-2 mRNA and protein expression in A549 cells

We determined the effects of hypertonic NaCl on COX-2 expression in A549 cells by using RT-PCR and Western blot analyses. A549 cells were treated with 100 mM NaCl, 200 mM urea, or 200 mM mannitol to the normal medium resulting in final osmolarity of 500 mosmol/kgH_2O. As shown in Fig. 1A, membrane-permeable urea did not affect the COX-2 protein levels, but the membrane-impermeable agents, NaCl and mannitol, increased COX-2 protein levels in the A549 cells. This observation indicates that the induction of COX-2 regulation in A549 cells in the presence of a high salt concentration occurs in response to cell volume changes by tonicity rather than by the osmolarity of the surrounding fluid. The A549 cells were treated with hypertonic NaCl for 0, 0.5, 1, 2, 4, 6, or 18 h. COX-2 mRNA was not detected after 0 or 0.5 h of the high salt treatment, but COX-2 mRNA was detected after 1 h and continued to accumulate until 18 h. To examine whether the induction of COX-2 mRNA was the result of increased transcription from the COX-2 promoter, we performed a reporter assay using a luciferase construct containing the full-length mouse COX-2 promoter. As shown in Fig. 1C, hypertonic NaCl significantly increased the luciferase activity of the full-length COX-2 promoter in the A549 cells.

We examined whether the hypertonic NaCl induction of COX-2 gene expression requires protein synthesis by pre-treating A549 cells for 2 h with or without cycloheximide (10 μg/ml), a protein synthesis inhibitor, and then incubating the cells in the presence or absence of 100 mM NaCl for 18 h. As a positive control, we performed RT-PCR for interleukin-8 (IL-8) [19]. The increased expression of COX-2 mRNA induced by the high-salt medium was not affected by cycloheximide pretreatment (Fig. 1D), although cycloheximide alone significantly increased IL-8 mRNA in the A549 cells. This result suggests that protein synthesis is not involved and indicates that the response is elicited by pre-existing transcription factor(s), possibly by NF-κB.

3.2. Hypertonic NaCl regulation of COX-2 is not mediated by NF-κB in A549 cells

The transcription factor NF-κB is important in the hypertonic NaCl regulation of COX-2 in kidney cells [9]. The binding of activated NF-κB to the COX-2 promoter region is critical for COX-2 transcriptional activation in a number of cell types [20–22]. NF-κB is a positive regulator of COX-2 expression in macrophages and colon carcinoma cell lines

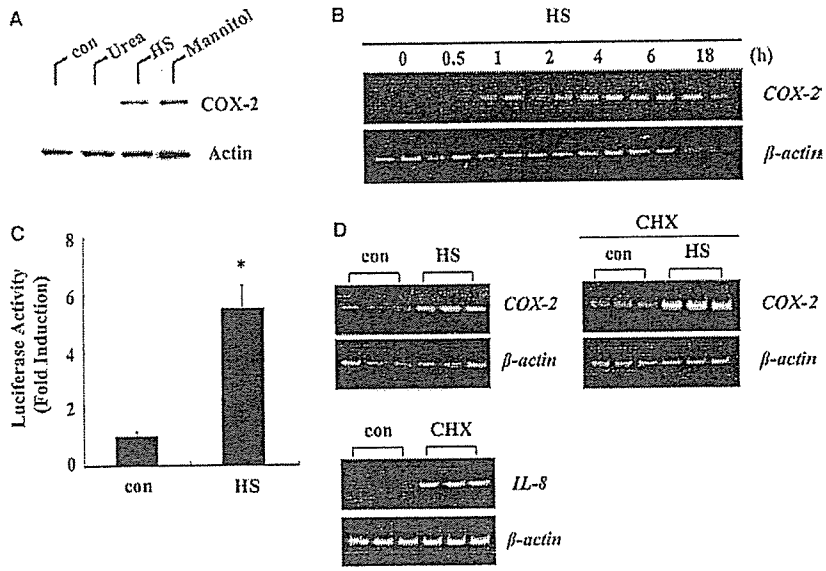

Fig. 1. Hypertonic NaCl increases COX-2 mRNA and protein expression in A549 cells. (A) A549 cells were treated with the indicated solutes (Urea, 200 mM urea; HS, 100 mM NaCl; mannitol, 200 mM mannitol) to the normal medium for 18 h. After incubation, the cell lysates were subjected to 10% SDS–PAGE and transferred to polyvinylidene difluoride membrane. Immunoblots were probed with a COX-2 antibody and reprobed with actin antibody. Bands were visualized by an ECL method, as described in Section 2. The immunoblot is representative of three independent experiments eliciting similar pattern. (B) A549 cells were treated with high salt (100 mM NaCl) for indicated time periods. Total RNA from A549 cells were analyzed for COX-2 mRNA expression by RT-PCR using specific primers as described in Section 2. Data presented are representative of two independent experiments showing similar trend. (C) A549 cells were transfected with COX-2-Luc and treated as indicated. After treatment, luciferase expression was determined as described in Section 2. Values represent the means ± S.E.M. ($N = 3$). *Represents $P < 0.05$. (D) A549 cells were pretreated with cycloheximide (10 μg/ml) for 2 h before incubation with 100 mM NaCl for 18 h. Total RNA from A549 cells were analyzed for COX-2 and IL-8 mRNA expression by RT-PCR assays. Data presented are representative of two independent experiments showing similar trend. con, untreated cells; HS, 100 mM NaCl; CHX, cycloheximide; IL-8, interleukin-8.

[23,24]. Studies have shown that IL-1β or lipopolysaccharide induces COX-2 expression via NF-κB activation in many cells including A549 cells [25–28]. To determine if NF-κB is involved in the hypertonic NaCl-induced COX-2 expression in A549 cells, we evaluated the effects of NF-κB inhibitors. We pretreated A549 cells with 5 μM MG132, a proteasome inhibitor that has been shown to prevent IκB degradation and thereby NF-κB activation [29], or 100 μM PDTC, followed by incubation in hypertonic NaCl for 18 h. As shown in Fig. 2A–C, neither MG132 nor PDTC inhibited COX-2 expression or promoter activation in cells exposed to hypertonic NaCl. No reduction in hypertonic NaCl induced COX-2 expression with MG132 or PDTC suggests that COX-2 activation occurs in the absence of NF-κB activation. Interestingly, we have consistently observed increased COX-2 protein expression with MG132 as compared with that of high salt (Fig. 2A, lanes 2 and 3). Similar results were observed in M-1 mouse cortical collecting duct cell (our unpublished results). We do not exactly understand how MG132 synergistically activates COX-2 in our model system. MG132 may activate upstream targets of hypertonic COX-2 activation by triggering other signaling transduction pathways independent of protein degradation [30].

To further determine the importance of NF-κB activation, we performed reporter assay using luciferase construct driven by the 5′-flanking region of the COX-2 promoter (−327/+59) containing NF-κB binding site. The COX-2 promoter (−327/+59) showed less than 2-fold activation in response to 100 mM NaCl (Fig. 2D). The 0.3 kb COX-2 promoter fragment with mutation at the NF-κB site (−223/−214) also had a marginal effect on luciferase expression (Fig. 2D). As a positive control for KBM, we used TPA [15]. These data suggest that NF-κB binding element is not critical in COX-2 upregulation by high salt in A549 cells. Furthermore, the hypertonic NaCl did not affect NF-κB-dependent luciferase expression in A549 cells. As a positive control for NF-κB-dependent gene transcription, we used YC-1, an activator of soluble guanylate cyclase, which initiates IKKα/β and NF-κB activation [31] (Fig. 2E). These observations indicate that NF-κB is not activated by hypertonic NaCl and imply that, although both salt and cytokines induce COX-2 expression, different signaling mechanisms exist for the hypertonic NaCl and cytokine induction of COX-2.

3.3. The hypertonic NaCl regulation of COX-2 in A549 cells is mediated by MEK1/2 and p38 MAPK

Study has shown that MAPK family members play a role in COX-2 gene expression induced by a hypertonic medium, which is crucial for cell survival under hyperosmotic shock [10]. The MAPK p38 is an essential component of the hypertonic signaling response pathway in mammals and is a major regulator in COX-2 upregulation [32–35]. To study the involvement of the MAPK pathway in the hypertonic NaCl induction of COX-2 expression in A549 cells, we evaluated the effects of p38 and MEK1/2 inhibitors on COX-2 expression. We pretreated A549 cells with SB203580 (a p38 MAPK inhibitor) or U0126 (a MEK1/2 inhibitor) for 30 min and then co-treated the cells with hypertonic NaCl for 18 h. The high-salt induction COX-2 mRNA and protein expression was

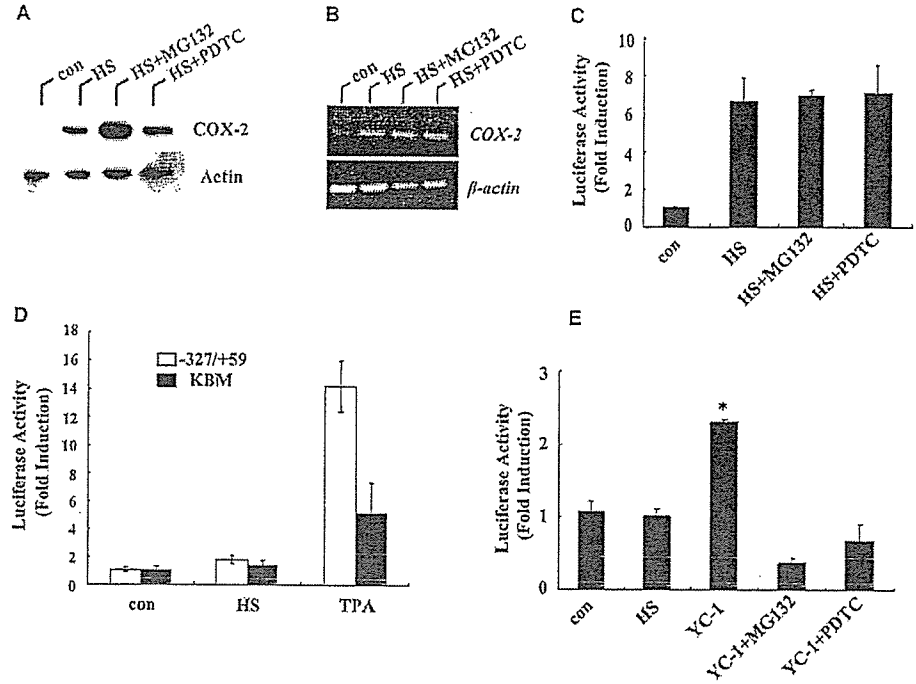

Fig. 2. NF-κB is not involved in hypertonic NaCl induction of COX-2 in A549 cells. (A) A549 cells were pretreated with PDTC (100 μM) or MG132 (5 μM) for 30 min before incubation with 100 mM NaCl for 18 h. Immunoblots were probed with a COX-2 antibody and reprobed with actin antibody, as described in Section 2. (B) A549 cells were pretreated with PDTC (100 μM) or MG132 (5 μM) for 30 min before incubation with 100 mM NaCl for 18 h. Total RNA from A549 cells were analyzed for COX-2 mRNA expression by RT-PCR assays, as described in Section 2. (C) A549 cells were transfected with COX-2-Luc and treated as indicated. After treatment, luciferase expression was determined as described in Section 2. Values represent the means ± S.E.M. ($N = 3$). (D) A549 cells were transfected with −327/+59 or KBM and treated as indicated. After treatment, luciferase expression was determined as described in Section 2. Values represent the means ± S.E.M. ($N = 3$). (E) A549 cells were transfected with the 3 × (NF-κB)tk-Luc and treated as indicated. After treatment, luciferase expression was determined. Values represent the means ± S.E.M. ($N = 3$). *Represents $P < 0.05$. con, untreated cells; HS, 100 mM NaCl. All experiments were repeated at least twice.

significantly blocked by SB203580 and U0126 (Fig. 3A and B). Furthermore, hypertonic NaCl-mediated full-length COX-2 promoter driven luciferase activity was partially inhibited by SB203580 and U0126 (Fig. 3C). It appears that p38 and MEK1/2 pathways affect not only at the level of transcription but also that of post-transcription in high-salt induction COX-2 expression in A549 cells. These results suggest that the activation of the p38 and MEK1/2 pathways is critical for the induction of COX-2 in A549 cells by a high-salt medium.

3.4. Dexamethasone inhibits hypertonic NaCl induction of COX-2 expression in A549 cells

Glucocorticoids regulate sodium uptake and fluid transport in both adult and fetal lungs, and studies have shown that a single dexamethasone injection increases AFC [36–40]. Dexamethasone suppresses COX-2 expression in the myelomonocytic leukemia cell line U937 [41]. The glucocorticoid receptor (GR) is involved in the tonic suppression of renal cortical COX-2 expression in animals [42]. To examine the possible

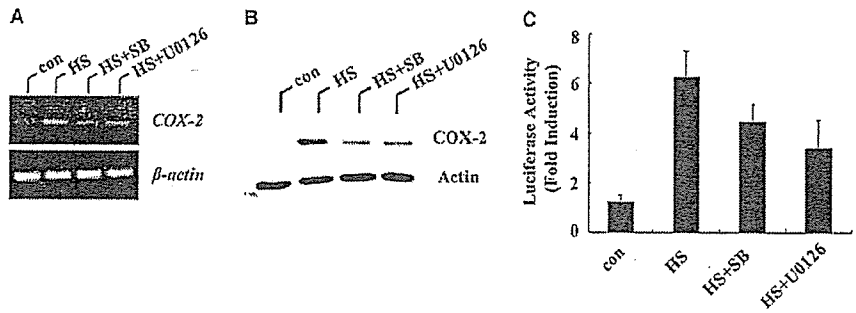

Fig. 3. MEK1/2 and p38 MAPK are involved in hypertonic NaCl induction of COX-2 in A549 cells. (A) A549 cells were pretreated with U1026 (10 μM) or SB203580 (10 μM) for 30 min before incubation with 100 mM NaCl for 18 h. Immunoblots were probed with a COX-2 antibody and reprobed with actin antibody, as described in Section 2. Data presented are representative of two independent experiments showing similar trend. (B) A549 cells were pretreated with U1026 (10 μM) or SB203580 (10 μM) for 30 min before incubation with 100 mM NaCl for 18 h. Total RNA from A549 cells were analyzed for COX-2 mRNA expression by RT-PCR assays, as described in Section 2. (C) A549 cells were transfected with COX-2-Luc and treated as indicated. After treatment, luciferase expression was determined as described in Section 2. Values represent the means ± S.E.M. ($N = 3$). con, untreated cells; HS, 100 mM NaCl; SB, SB203580. The immunoblot is representative of three independent experiments eliciting similar pattern.

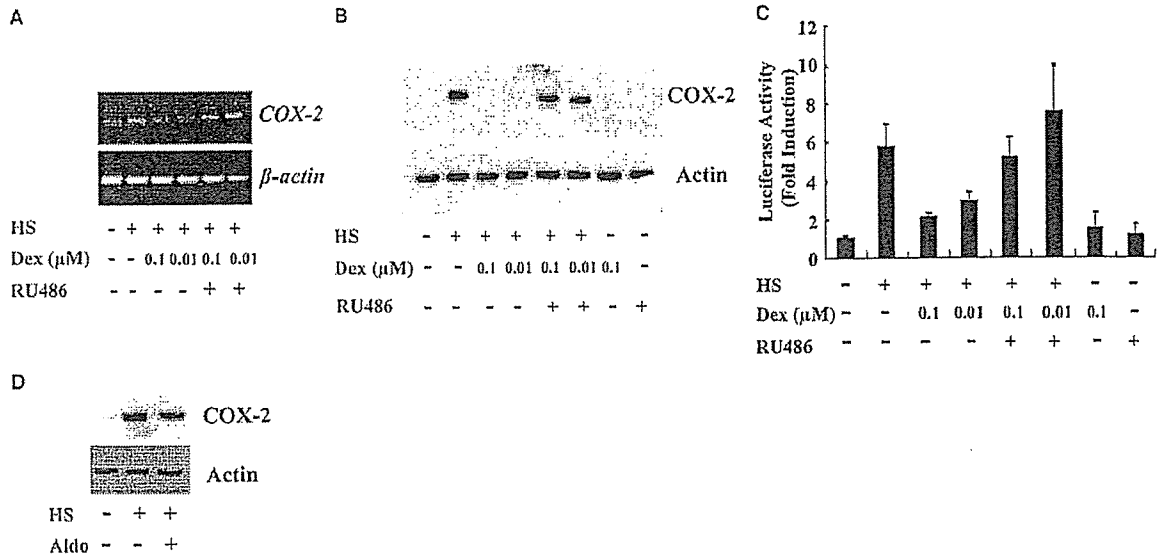

Fig. 4. Dexamethasone inhibits hypertonic NaCl induction of COX-2 in A549 cells. (A) A549 cells were pretreated with dexamethasone (0.01 or 0.1 μM) and/or RU486 (1 μM) for 1 h before incubation with 100 mM NaCl for 18 h. Total RNA from A549 cells were analyzed for COX-2 mRNA expression by RT-PCR assays, as described in Section 2. Data are representative of two independent experiments. (B) A549 cells were pretreated with dexamethasone (0.01 or 0.1 μM) and/or RU486 (1 μM) for 1 h before incubation with 100 mM NaCl for 18 h. Immunoblots were probed with a COX-2 antibody and reprobed with actin antibody, as described in Section 2. Blots are representative of four independent experiments showing similar pattern. (C) A549 cells were transfected with COX-2-Luc and treated as indicated. After treatment, luciferase expression was determined as described in Section 2. Experiments were repeated four times. (D) A549 cells were pretreated with aldosterone (0.1 μM) for 1 h before incubation with 100 mM NaCl for 18 h. Immunoblots were probed with a COX-2 antibody and reprobed with actin antibody. Blots are representative of two independent experiments showing similar pattern. Values represent the means ± S.E.M. ($N = 3$). *Represents $P < 0.05$. con, untreated cells; HS, 100 mM NaCl; Dex, dexamethasone; Aldo, aldosterone. Experiments were repeated twice.

role of the GR in the hypertonic NaCl induction of COX-2 in A549 cells, we pre-incubated cells with dexamethasone and/or the GR antagonist RU486 for 1 h and co-treated with NaCl. Neither dexamethasone nor RU486 in the absence of high salt had an effect on the COX-2 protein level or promoter activity. However, dexamethasone did block the hypertonic NaCl-induced COX-2 mRNA (Fig. 4A) and protein (Fig. 4B) expression, and COX-2 promoter activity (Fig. 4C). The suppressive effect of dexamethasone was antagonized by the GR antagonist RU486, suggesting that the response is mediated by the GR and glucocorticoid-specific. To further determine the specificity of the inhibitory effect of dexamethasone on salt-induced COX-2 regulation, we examined whether aldosterone exhibits similar suppressive effects. Cells were pre-incubated with aldosterone and/or the mineralocorticoid receptor antagonist spironolactone for 1 h and were co-treated with NaCl for 18 h. As shown in Fig. 4D, the application of aldosterone did not have an effect on the hypertonic NaCl induction of COX-2 protein expression in A549 cells. As a positive control, we performed an αENaC promoter-driven reporter gene assay; aldosterone significantly increased αENaC-dependent reporter gene transcription, and spironolactone blocked this induction (data not shown). These results indicate that dexamethasone inhibits hypertonic NaCl-induced COX-2 regulation through the GR in A549 cells.

4. Discussion

We studied the induction of COX-2 by hypertonic NaCl in lung epithelial A549 cells and observed that NaCl increases COX-2 expression at the level of transcription. Our study suggests that p38 and MEK1/2, and not NF-κB, are involved in the signal transduction leading to the expression of COX-2 induced by hypertonic NaCl. The NaCl induction of COX-2 in the lung cells could be a non-specific inflammatory-related response, but urea, a hyperosmotic agent that can promote cell lysis and can be irritating to cells, did not induce COX-2 in our study. COX-2 induction was observed in response to an increase in tonicity. The hypertonic NaCl induction of COX-2 has been studied most intensively in kidney tissue [6,7,9,10,43]. The expression of COX-2 in the kidney is tissue-specific; COX-2 is downregulated in the cortex and upregulated in the medulla [44]. Despite intensive in vivo and in vitro studies, the mechanism of the differential regulation in the kidney has not been elucidated. Our results show that hypertonic NaCl activates COX-2 expression in lung epithelial cells, as occurs in rat kidney medulla and inner medullary collecting duct cells [9,10,43].

The first candidate we investigated as a transcription factor responsible for COX-2 gene activation in lung epithelial cells was NF-κB. Studies have previously shown that hypertonic stress activates an NF-κB-COX-2-linked survival mechanism in renal medullary interstitial cells [43]. Other studies have shown that the inhibition of glycogen synthase kinase-3β protects renal cells from hypertonic stress via the induction of the NF-κB-COX-2-dependent pathway [9]. However, in contrast to the situation in the kidney medulla, the hypertonic activation of COX-2 in lung epithelial cells was not dependent on NF-κB. In addition, it appears that part of the signal transduction pathway leading to COX-2 activation is shared but is not identical with other stimuli, such as LPS and cytokines. The NaCl induction of COX-2 was inhibited by a glucocorticoid,

which agrees with the observations made in other studies of LPS and cytokines [45–47], while dexamethasone suppressed LPS- and IL-1β-induced COX-2 regulation in a NF-κB-dependent manner. Recent studies have shown that the inhibition of granulocyte-macrophage colony-stimulating factor by dexamethasone is independent of NF-κB [48,49], and the inhibition of NF-κB cannot account for all the repressive effects of dexamethasone on inflammatory genes such as COX-2 [50]. The transcription factor involved in the hypertonic NaCl activation of COX-2 remains to be identified, but it is most likely a pre-existing protein(s) rather than a newly synthesized protein, as shown by the cycloheximide experiments. It is possible that the factor is specific to the lung; however, we have observed the hypertonic NaCl activation of COX-2 in other cells, such as vascular smooth muscle (data not shown), which indicates that the response is not restricted to lung and kidney tissues. A few transcription factors activated by hypertonicity are known. One well-known example is tonicity-responsive enhancer (TonE) binding protein (TonEBP). TonEBP is a member of the Rel family of transcriptional activators that include NF-κB and nuclear factor of activated T cells [51]. Studies have previously shown that the hypertonic induction of COX-2 mRNA is not reduced by the expression of DN-TonEBP [52]. However, additional studies are needed to confirm that the hypertonic induction of COX-2 is independent of TonEBP. The identification of the transcription factor responsible for the hypertonic NaCl induction of COX-2 would greatly enhance our understanding of the hypertonicity-triggered signal transduction pathway that leads to COX-2 activation.

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