Involved of Ras/Raf-1/p44/42 MAPK in YC-1-induced cyclooxygenase-2 expression in human pulmonary epithelial cells

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Our previous study demonstrated that 3-(5-hydroxymethyl-2-furyl)-1-benzylindazole (YC-1) might activate the soluble guanylate cyclase (sGC)/cGMP/protein kinase G (PKG) pathway to induce cyclooxygenase-2 (COX-2) expression in human pulmonary epithelial cells (A549). In this study, we further investigated the role of Raf-1 in YC-1-induced nuclear factor-xB (NF-xB) activation and COX-2 expression in A549 cells. YC-1-induced COX-2 expression was attenuated by a Raf-1 inhibitor (GW 5074) in a concentration-dependent manner. Treatment of A549 cells with YC-1 or 8-bromo-cGMP, a cell-permeable cGMP analogue, induced Raf-1 Ser338 phosphorylation in a time-dependent manner. YC-1-mediated Raf-1 activation was inhibited by a sGC inhibitor (ODQ), a PKG inhibitor (KT-5823), a Ras inhibitor (manumycin A), a dominant negative Ras mutant (RasN17), a protein kinase C-α (PKC-α) inhibitor (Ro 32-0432), and a phosphoinositide-3-OH-kinase (PI3K) inhibitor (LY 294002). Pretreatment of A549 cells with either manumycin A or GW 5074 attenuated YC-1-induced p44/42 MAPK activation. The YC-1-mediated increase in IKKs/β activation and kβ-luciferase activity were attenuated by GW 5074, a MAPK/ERK kinase (MEK) inhibitor (PD 98059), and an ERK2 inhibitor (AG 126). Furthermore, YC-1-induced COX-2 promoter activity was also inhibited by GW 5074, PD 98059, and AG 126. These results indicate that YC-1 might activate the sGC/cGMP/PKG pathway to elicit Ras/Raf-1/p44/42 MAPK activation, which in turn induces IKKs/β and NF-xB activation, and ultimately causes COX-2 expression in A549 cells. Moreover, PKC-α and PI3K signal might be involved in YC-1-induced Raf-1 activation.

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1. Introduction

Cyclooxygenase (COX), also referred to as prostaglandin (PG) endoperoxide synthases, catalyzes the rate-limiting step in the synthesis of PGs, a potent group of autocrine and paracrine lipid mediators [1,2] that have been implicated in many normal cellular and pathophysiological processes, such as inflammation, edema, bronchoconstriction, platelet aggregation, fever, and hyperalgesia [1–3]. Two forms of COX have been identified: a constitutively expressed form, COX-1, and an inducible form, COX-2. Both isoforms catalyze the conversion of arachidonic acid and O2 to generate PGH2, which serves as a common precursor for PGs, prostacyclin, and thromboxanes [4]. The two COX isoforms are encoded by distinct genes. COX-1 is a housekeeping gene, generally thought to produce prostaglandins which serve to maintain cellular homeostasis, and is known to be constitutively expressed in many cell types, including endothelial cells, platelets, and gastric mucosa [5]. In contrast, COX-2, first identified as an immediate early response gene [6], is thought to mediate inflammatory events and shows low basal expression, but is rapidly induced by proinflammatory mediators [7]. A549 cells, a human pulmonary epithelial carcinoma cell line with type II alveolar epithelial cell differentiation, show features similar to those of type II alveolar epithelial cells. Airway epithelial cells play an active role in inflammation by producing various cytokines and eicosanoids [8]. It has been demonstrated...
that proinflammatory cytokines, such as IL-1β, increase COX-2 expression and PGE₂ release in human A549 cells [7].

Ras has been linked to numerous effector pathways that lead to the induction of diverse physiological and pathological responses. These pathways regulate cell proliferation, cytoskeletal changes, and the release of proinflammatory mediators [9]. An important class of Ras effectors is the mitogen-activated protein kinase (MAPK) family. The “classic” Ras-mediated pathway involves binding and activation of the serine/threonine kinase, Raf-1, which in turn activates the dual specificity kinase, MAPK/ERK kinase (MEK), resulting in activation of p44/42 MAPKs [10], p44/42 MAPKs phosphorylate a number of target proteins, including transcription factors and intracellular enzymes [11]. Activated Ras binds to Raf-1 with high affinity, but does not directly alter the catalytic activity of Raf-1 [12]. Rather, it relocalizes Raf-1 from the cytosol to the plasma membrane where a multistep activation process takes place. Although the initial interaction between the effector domain of Ras and the Ras-binding domain (RBD) of Raf-1 is both necessary and sufficient for membrane translocation, a secondary interaction between the Raf-1 cysteine-rich domain (CRD) and possibly the far-nestylated tail of Ras is required for activation to ensue [13,14]. It is well documented that the Ras/Raf-1/MAPK signaling pathway is necessary for transcriptional induction of COX-2 by several kinds of stimuli [15–17]. Moreover, several studies have indicated that Raf-1 regulates nuclear factor-kB (NF-κB) activation and leads to cell transformation [18,19].

3-(5′-Hydroxymethyl-2′-furyl)-1-benzylindazole (YC-1) has been identified as an activator of soluble guanylate cyclase (sGC), and was shown to increase the intracellular cGMP concentration in platelets [20]. The cGMP-increasing effect of YC-1 has been reported to mediate tracheal relaxation [21] and vascular protection [22]. It was further demonstrated that YC-1 not only stimulates sGC but also inhibits cGMP-hydrolyzing phosphodiesterase in human platelets [23]. Previously, we demonstrated that in human pulmonary epithelial (A549) cells, YC-1 might activate the sGC/cGMP/protein kinase G (PKG) pathway to induce COX-2 expression via two pathways: the protein kinase C-α (PKC-α)/p44/p42 MAPK cascade [24] and the Ras/phosphoinositide-3-ÓH-kinase (PI3K)/Akt/IκB kinases α/β (IκKα/κ)/NF-κB cascade [25]. However, the molecular mechanism underlying YC-1-induced COX-2 expression still remains to be determined. Therefore, the objective of this study was to identify the role of Raf-1 and relationships among Ras, PI3K, PKC-α, Raf-1, p44/42 MAPK, and IκKα/κ in YC-1-induced NF-κB activation and COX-2 expression in human pulmonary epithelial cells. Our results show that YC-1 might activate the sGC/cGMP/PKG pathway to elicit Ras/Raf-1/p44/42 MAPK activation, which in turn induces activations of IκKα/κ and NF-κB, ultimately causing COX-2 expression in human pulmonary epithelial cells (A549). Moreover, PKC-α and PI3K signal might be involved in YC-1-induced Raf-1 activation.

2. Materials and methods

2.1. Materials

YC-1, 8-bromo-cGMP, 1H-(1,2,4)oxadiazolо[4,3-a]quinozalin-1-one (ODQ), KT-5823, manumycin A, Ro 32-0432, LY 294002, PD 98059, and AG 126 were purchased from Calbiochem-Novabiochem (San Diego, CA). GW 5074 was purchased from Tocris (Avonmouth, UK). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12, fetal calf serum (FCS), and penicillin/streptomycin were purchased from Life Technologies (Gaithersburg, MD). An antibody specific for COX-2 was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibodies specific for Raf-1, p44/42 MAPK, IκB kinase (IKK) α/β, and a mouse monoclonal antibody specific for phospho-p44/42 MAPK, as well as horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for phospho-Raf-1 (Ser338) and phospho-IκKα (Ser180)/IκKβ (Ser181) were purchased from New England Biolabs (Beverly, MA). An antibody specific for α-tubulin was purchased from Oncogene Science (Cambridge, UK). pGL2-ELAM-Luc, which is under the control of a single NF-κB binding site, and pBK-CMV-Lac Z were provided by Prof. W.-W. Lin (National Taiwan University, Taipei, Taiwan). A human COX-2 promoter-luciferase construct, pG5459 (−459/+9) which contains two κB binding sites, was a kind gift from Prof. C.-C. Chen (National Taiwan University, Taipei, Taiwan). A dominant negative Ras mutant (RasN17) was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-mouse immunoglobulin G (IgG)-conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories (West Grove, PA). 4-Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Boehringer Mannheim (Mannheim, Germany). The enhanced chemiluminescence detection agent was purchased from PerkinElmer Life Sciences (Boston, MA). All chemicals for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Cell culture

A549 cells, a human pulmonary epithelial carcinoma cell line with type II alveolar epithelial cell differentiation, were obtained from the American Type Culture Collection (Livingstone, MT) and grown in DMEM/Ham's F-12 nutrient mixture containing 10% FCS and penicillin/streptomycin (50 U/ml) in a humidified 37°C incubator. After cells had grown to confluence, they were disaggregated in a trypsin solution, washed 125 × g for 5 min, resuspended, and then subcultured according to standard protocols. Cells were seeded onto 6 cm dishes for immunoblotting, or onto 12-well plates for κB-luciferase assays.

2.3. Protein preparation and Western blotting

To determine the levels of COX-2, α-tubulin, phospho-Raf-1 (Ser338), Raf-1, phospho-p44/42 MAPK, p44/42 MAPK, phospho-IκKα (Ser180)/IκKβ (Ser181), and IκKα/κ in A549 cells, proteins were extracted, and Western blot analysis was performed as previously described [26]. Briefly, A549 cells were cultured in 6 cm dishes. After reaching confluence, cells were treated with YC-1 or 8-bromo-cGMP, or pretreated with specific inhibitors as indicated followed by YC-1. To assay the effect of a dominant-negative RasN17 (RasN17) cells were transfected with RasN17 for 24 h before YC-1 treatment. After incubation, cells were washed with PBS, incubated with extraction buffer (10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5% NP-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin) with gentle shaking, and then centrifuged at 12,500 rpm for 30 min. The cell extract was then boiled in sample buffer (100 mM Tris (pH 6.8), 20% glycerol, 4% SDS, and 0.2% bromphenol blue) at a ratio of 1:1. Electrophoresis was performed using 10% SDS-polyacrylamide gels (2 h, 110 V, 40 mA, 40 µg protein per lane). Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (2 h, 40 V), treated with 5% fat-free milk powder to block the non-specific IgGs, and incubated for 2 h with specific antibodies for COX-2, α-tubulin, phospho-Raf-1 (Ser338), Raf-1, phospho-p44/42 MAPK, p44/42 MAPK, phospho-IκKα (Ser180)/IκKβ (Ser181), and IκKα/κ. The blot was then incubated with anti-mouse or -rabbit IgG linked to alkaline phosphatase or horseradish peroxidase for 2 h. Subsequently, the immunoreactivity was detected with NBT/BCIP as a substrate or by enhanced chemiluminescence. Quantitative data were obtained...
using a computing densitometer with Image-Pro plus software (Media Cybernetics, Silver Spring, MD).

2.4. Transfection and luciferase reporter assays

A549 cells at 2.5 × 10⁵ were seeded into 12-well plates. For the COX-2 promoter and κB-luciferase assays, cells were transfected on the following day with the Lipofectamine plus™ reagent containing 1 μg of pGS459 (−459/+9) or 0.5 μg of pGL2-ELAM-Luc, respectively, and 1 μg of pBK-CMV-Lac Z for 24 h. After incubation, the medium was aspirated and replaced with fresh DMEM/Ham’s F12 containing 10% FCS. Cells were then stimulated with YC-1 for another 24 h before harvesting. To assay the effects of various inhibitors, drugs were added to the cells 30 min before YC-1 stimulation. Luciferase activity was determined with a luciferase assay system (Promega) and normalized on the basis of Lac Z expression. The level of induction of luciferase activity was determined as a ratio in comparison to cells with no stimulation.

2.5. Statistical analysis

Results are presented as the mean ± S.E.M. from at least three independent experiments. One-way analysis of variance (ANOVA) followed by Bonferroni’s multiple-range test, when appropriate, was used to determine the statistical significance of the difference between means. A p-value of <0.05 was taken as statistically significant.

3. Results

3.1. Involvement of Raf-1 in YC-1-induced COX-2 expression

We previously demonstrated that YC-1 might activate the sGC/cGMP/PKG pathway to induce COX-2 expression in A549 cells through two separate pathways: the PKC-ε/p44/42 MAPK cascade [24] and the Ras/PI3K/Akt/IKKα/β/NF-κB cascade [25]. In this study, we examined whether the main downstream effector of Ras, Raf-1, is also involved in the signal transduction pathway leading to COX-2 expression caused by YC-1, using the Raf-1 inhibitor, GW 5074 [27]. Pretreatment of cells for 30 min with GW 5074 (0.1–10 nM) attenuated YC-1-induced COX-2 expression in a concentration-dependent manner. GW 5074 (10 nM) inhibited YC-1-induced COX-2 expression by 57 ± 12% (Fig. 1). Since Ras-induced Ser338 phosphorylation...
of Raf-1 causes enzymatic activation [28], the antibody specific against phosphorylated Raf-1 (Ser338) was used to examine Raf-1 phosphorylation, an index of kinase activation. Treatment of A549 cells with 50 μM YC-1 or 30 μM 8-bromo-cGMP, a cell-permeable cGMP analogue, induced Raf-1 Ser338 phosphorylation in a time-dependent manner, beginning at 5 or 10 min after treatment, respectively, and reaching a maximal level at 10–20 min after treatment (Fig. 2A and B). The protein level of Raf-1 was not affected by YC-1 or 8-bromo-cGMP treatment (Fig. 2A and B). YC-1-induced Raf-1 activation was markedly inhibited by pretreatment of cells for 30 min with 30 μM ODQ (an sGC inhibitor) and 3 μM KT-5823 (a PKG inhibitor) by 64 ± 9% and 78 ± 13%, respectively (Fig. 2C). None of these treatments had any significant effect on the Raf-1 protein level (Fig. 2C).

To further confirm the role of Ras in YC-1-induced Raf-1 activation in A549 cells, a Ras inhibitor (manumycin A) and a dominant negative Ras mutant (RasN17) were used. Pretreatment of cells with manumycin A (1–10 μM) or transfection of cells with RasN17 (0.5 and 1 μg) for 24 h markedly attenuated YC-1-induced Raf-1 activation. Manumycin A (10 μM) and RasN17 (1 μg) almost completely abolished YC-1-induced Raf-1 activation (Fig. 3A and B). Our previous study revealed that PKC-α is involved in YC-1-induced p44/42

Fig. 3. Involvement of Ras, PKC-α, but not PI3K in YC-1-induced Raf-1 activation in A549 cells. Cells were pretreated with various concentrations of manumycin A (Manu) for 24 h (A), Ro 32-0432 (Ro) or LY 294002 (LY) for 30 min (C) and (D), or transfected with a dominant negative Ras mutant (RasN17) for 24 h (B) before incubation with 50 μM YC-1 for 20 min. Cells were then prepared and subjected to Western blot analysis using antibodies specific for phosphorylated Raf-1 (Ser338) or Raf-1 as described in Section 2. Equal loading in each lane is demonstrated by similar intensities of Raf-1. The extent of Raf-1 activation was quantitated using a densitometer with Image-Pro plus software. Data are representative of three independent experiments which gave essentially identical results. Results are expressed as the mean ± S.E.M. (n = 3). * p < 0.05, compared to the YC-1-treated group.
MAPK activation [24]. To examine whether PKC-α is involved in YC-1-induced Raf-1 activation, the PKC-α inhibitor Ro 32-0432 was used. As shown in Fig. 3C, the YC-1-induced Raf-1 activation was concentration-dependently inhibited by pretreatment of cells with Ro 32-0432 (1–10 μM). Furthermore, we examined whether PI3K is also involved in YC-1-induced Raf-1 activation, using the PI3K inhibitor, LY 294002. Pretreatment of cells for 30 min with LY 294002 (3–10 μM) attenuated YC-1-induced Raf-1 activation. When cells treated with 10 μM LY 294002, YC-1-induced Raf-1 activation was inhibited by 83 ± 13% (Fig. 3D). None of these treatments had any significant effect on the Raf-1 protein level (Fig. 3).

3.2. Involvement of Ras and Raf-1 in YC-1-induced p44/42 MAPK activation

Since activation of MAPKs requires phosphorylation at the threonine and tyrosine residues, an immunoblot analysis was performed to examine MAPK phosphorylation using anti-phospho-p44/42 MAPK-specific antibodies. Our previous results showed that YC-1 can induce p44/42 MAPK activation in A549 cells [24]. In the present study, pretreatment of the cells with either manumycin A (3 μM) for 24 h or GW 5074 (10 nM) for 30 min inhibited YC-1-induced p44/42 MAPK activation by 65 ± 2% and 47 ± 4%, respectively (Fig. 4). None of these treatments had any significant effect on p44/42 MAPK expression (Fig. 4).

![Fig. 4. Effects of manumycin A (Manu) and GW 5074 (GW) on YC-1-induced p44/42 MAPK activation in A549 cells. Cells were pretreated with 3 μM manumycin A for 24 h or 10 nM GW 5074 for 30 min before incubation with 50 μM YC-1 for 30 min. Cell lysates were prepared and subjected to Western blot analysis using antibodies specific for phosphorylated p44/42 MAPK (p44/42-p) or p44/42 MAPK (p44/42) as described in Section 2. Equal loading in each lane is demonstrated by similar intensities of p44/42. The extent of p44/42 MAPK activation was quantitated using a densitometer with Image-Pro plus software. Data are representative of three independent experiments which gave essentially similar results. Results are expressed as the mean ± S.E.M. (n = 3). *p < 0.05, compared to the YC-1-treated group.](image-url)

![Fig. 5. Effects of various inhibitors on YC-1-induced increases in IKKα/β activation and kβ-luciferase activity in A549 cells. (A) Cells were pretreated with 10 nM GW 5074 (GW) or 30 μM PD 98059 (PD) for 30 min before incubation with 50 μM YC-1 for 30 min. Cell lysates were prepared and subjected to Western blot analysis using antibodies specific for phosphorylated IKKα (Ser180)/IKKβ (Ser181) or IKKα/β as described in Section 2. Equal loading in each lane is demonstrated by similar intensities of IKKα/β. The extent of IKKα/β activation was quantitated using a densitometer with Image-Pro plus software. Data are representative of three independent experiments which gave essentially similar results. Results are expressed as the mean ± S.E.M. (n = 3). *p < 0.05, compared to the YC-1-treated group. (B) Cells were transiently transfected with 0.5 μg of pGL2-ELAM-Luc and 1 μg of pBK-CMV-Lac Z for 24 h, and then pretreated with 10 nM GW 5074 or 50 μM AG 126 for 30 min, before incubation with 25 μM YC-1 for 24 h. Cells were then harvested for the kβ-luciferase assay as described in Section 2. Results are expressed as the mean ± S.E.M. (n = 3). *p < 0.05, compared to the YC-1-treated group.](image-url)
4. Discussion

A G 1 2 6b y5 1 % sites in the COX-2 promoter. Treatment with 50 nM GW 5074 was inhibited by 10 nM GW 5074, 30 μM PD 98059, or 50 μM AG 126 for 30 min, before incubation with 25 μM YC-1 for 24 h. Cells were then harvested for the COX-2 promoter-luciferase assay as described in Section 2. Results are expressed as the mean ± S.E.M. (n = 3).

3.3. Involvement of Raf-1 and p44/42 MAPK in YC-1-induced increases in IKKα/β phosphorylation, κB-luciferase activity, and COX-2 promoter luciferase activity

Our previous studies indicated that YC-1 might induce IKKα/β and activation of its downstream effector, NF-κB, which in turn causes COX-2 expression in A549 cells [25]. In this study, pretreatment of cells for 30 min with GW 5074 (10 nM) or a MEK inhibitor (PD 98059, 30 μM) attenuated the YC-1-induced IKKα/β phosphorylation by 47 ± 8% and 62 ± 9%, respectively (Fig. 5A and B). GW 5074 (10 nM) and 30 μM PD 98059 also inhibited the basal level of IKKα/β phosphorylation by 33 ± 14% and 29 ± 16%, respectively (Fig. 5A). Similarly, pretreatment of cells for 30 min with GW 5074 (10 nM) or AG 126 (50 μM) also markedly inhibited the YC-1-induced increase in κB-luciferase activity by 40 ± 7% and 64 ± 9%, respectively (Fig. 5B). To further confirm the roles of Raf-1 and p44/42 MAPK in YC-1-induced NF-κB activation, transient transfections were performed using the human COX-2 promoter-luciferase construct, pG5459 (-459/-9). This construct contains both upstream (-441/-438) and downstream (-223/-234) NF-κB sites in the COX-2 promoter. Treatment with 50 μM YC-1 led to a 201 ± 9% increase in COX-2 promoter-luciferase activity; this effect was inhibited by 10 nM GW 5074, 30 μM PD 98059, and 50 μM AG126 by 51 ± 7%, 83 ± 6%, and 79 ± 2%, respectively (Fig. 6).

4. Discussion

Our previous study showed that YC-1, an activator of sGC, activated PKG through an upstream sGC/cGMP pathway to elicit PKC-α activation, which in turn initiated p44/42 MAPK activation, and finally induced COX-2 expression in human pulmonary epithelial cells (A549) [24]. Furthermore, we also demonstrated that YC-1 might activate the sGC/cGMP/PKG pathway to induce Ras and PI3K/Akt activation, which in turn initiates increases in IKKα/β activity and NF-κB activation, ultimately inducing COX-2 expression [25]. In the present study, we further demonstrate that the Ras/Raf-1/p44/42 MAPK pathway might also be involved in the YC-1-induced activations of IKKα/β and NF-κB, and the expression of COX-2 protein.

The MAPK family is an important downstream effector of Ras. The “classic” Ras-mediated pathway involves binding and activation of the serine/threonine kinase, Raf-1, which in turn activates the dual specificity kinase, MAPK/ERK kinase (MEK), resulting in activation of p44/42 MAPKs [10]. Recently, several reports have indicated that the Ras/Raf-1/MAPK signaling pathway is necessary for the transcriptional induction of COX-2 [15,17]. In this study, we found that GW 5074 (a Raf-1 inhibitor) and AG 126 (a p44/42 MAPK inhibitor) inhibited YC-1-induced increases in COX-2 expression and COX-2 promoter activity. Furthermore, treatment of A549 cells with YC-1 caused activation of Raf-1 and p44/42 MAPK. These results suggested that activations of Raf-1 and p44/42 MAPK are very important for COX-2 induction caused by YC-1. Furthermore, YC-1-induced Raf-1 activation was inhibited by ODQ (an sGC inhibitor), KT-5823 (a PKG inhibitor), manumycin A (a Ras inhibitor), a dominant negative Ras mutant (RasN17). Moreover, 8-bromo-cGMP, a cell-permeable cGMP analogue, also induced Raf-1 phosphorylation. Recently, we also found that YC-1-induced Ras activation occurs downstream of the signals of sGC and PKG [25]. These results indicate that YC-1 might activate the sGC/cGMP/PKG pathway to induce Ras activation, which in turn causes Raf-1 activation in A549 cells. Furthermore, we demonstrated that YC-1-induced p44/42 MAPK activation was inhibited by manumycin A and GW 5074, suggesting that activations of Ras and Raf-1 occur upstream of YC-1-induced p44/42 MAPK activation.

In a previous study, we found that phorbol 12-myristate 13-acetate (PMA), a PKC activator, might activate PKC to elicit activation of the Ras/Raf-1/ERK1/2 pathway, which in turn induces COX-2 expression in A549 cells [29]. Recently, lead acetate (Pb) has been reported to activate PKCα and subsequent the Ras/Raf-1/MEK1/2/ERK1/2 signaling cascade in C3 human non-small-cell lung adenocarcinoma cells [30]. In this study, we found that Ro 32-0432 (a PKC-α inhibitor) inhibited YC-1-induced Raf-1 activation. Previous study has also demonstrated that in human skin fibroblasts, elastin peptides might activate ERK1/2 via PI3K/Akt-dependent Raf-1 signaling pathway [31]. In this study, we showed that YC-1-induced Raf-1 activation was inhibited by LY 294002 (a PI3K inhibitor). These results indicate that in addition to Ras, PKC-α and PI3K signal might be involved in YC-1-induced Raf-1 activation in A549 cells.

NF-κB is the most important transcription factor that regulates COX-2 expression. Activation of NF-κB plays a pivotal role in regulating both the inflammatory response and immunity [32,33]. Our previous study demonstrated that in A549 cells, YC-1-induced increases in κB-luciferase activity, COX-2 promoter activity, and COX-2 expression were attenuated by an NF-κB blocker (PDTC) and a dominant negative IkBα mutant (IkBαM), indicating that NF-κB activation is necessary for YC-1-induced COX-2 expression in A549 cells [25]. Previous reports showed that Ras enhances NF-κB transcriptional activity through the Raf-1-dependent p44/42 MAPK pathway in NIH-3T3 cells [18]. A previous report showed that in transformed liver epithelial cells, Ras and Raf-1 lead to constitutive activation of NF-κB through the IKKα/β complex [34]. In this study, we found that the YC-1-induced IKKα/β activation was inhibited by GW 5074 and a MEK inhibitor (PD 98059). Furthermore, the YC-1-mediated increase in κB-luciferase activity was inhibited by GW 5074 and AG 126. Previous studies also found that YC-1-induced increases in IKKα/β activation and κB-luciferase activity were inhibited by manumycin A and a dominant negative Ras mutant (RasN17) [25]. These results indicate that activation of the Ras/Raf-1/p44/42 MAPK pathway is involved in YC-1-induced NF-κB activation through an increase in IKKα/β activity.

In conclusion, YC-1 might activate the sGC/cGMP/PKG pathway to elicit Ras/Raf-1/p44/42 MAPK activation, which in turn initiates IKKα/β and NF-κB activation, and finally induces COX-2 expression in human pulmonary epithelial cells (A549). Moreover, PKC-α and PI3K signal might be involved in YC-1-induced Raf-1 activation.
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