Pharmacological Research 60 (2009) 247–253

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

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

Ming-Shyan Chang[a], Bing-Chang Chen[b], Chih-Ming Weng[a], Wen-Sen Lee[a], Chien-Huang Lin[a,c,d,*]

[a] Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan
[b] School of Respiratory Therapy, College of Medicine, Taipei Medical University, Taipei, Taiwan
[c] Taipei Medical University, Wang-Fang Hospital, Taipei, Taiwan
[d] Taipei Medical University, Shuang-Ho Hospital, Taipei County, Taiwan

ARTICLE INFO

Article history:
Received 8 December 2008
Received in revised form 10 March 2009
Accepted 24 March 2009

Keywords:
YC-1
Cyclooxygenase-2
Ras
Raf-1
p44/42 MAPK
NF-κB
IKKα/β
A549 cells

ABSTRACT

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-κB (NF-κB) 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 an 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 IKKα/β activation and κB-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 IKKα/β and NF-κB 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.

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 O_2 to generate PGH_2, 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

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; cGMP, 3′,5′-cyclic guanosine monophosphate; COX, cyclooxygenase; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FCS, fetal calf serum; ODQ, 1H-(1,2,4)oxadiazolo[4,3-a]quinozalin-1-one; HEPES, 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NF-κB, nuclear factor-κB; NBT, 4-nitro blue tetrazolium; NP-40, nonidet P-40; PMSF, phenylmethylsulphonyl fluoride; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; PKC-α, protein kinase C-α; PKG, protein kinase G; PGE_2, prostaglandin E_2; PGs, prostaglandins; PI3K, phosphoinositide-3-OH-kinase; SDS, sodium dodecylsulfate; sGC, soluble guanylate cyclase.

* Corresponding author at: Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan. Tel.: +886 2 27361661x7100; fax: +886 2 27399145.
E-mail address: chlin@tmu.edu.tw (C.-H. Lin).

1043-6618/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phrs.2009.03.015

that proinflammatory cytokines, such as IL-1β, increase COX-2 expression and PGE_2 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 farnesylated 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-κB (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)/p44/42 MAPK cascade [24] and the Ras/phosphoinositide-3-OH-kinase (PI3K)/Akt/IκB kinases α/β (IKKα/β)/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 IKKα/β 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 IKKα/β 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)oxadiazolo[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-IKKα (Ser180)/IKKβ (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, pGS459 (−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-indolylphosphate (BCIP) were purchased from Boehringer Mannheim (Mannheim, Germany). The enhanced chemiluminescence detection agent was purchased from PerkinElmer Life Sciences (Boston, MA). All materials 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 in DMEM/Ham's F-12 supplemented with 10% FCS, centrifuged at 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-IKKα (Ser180)/IKKβ (Ser181), and IKKα/β 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% bromophenol 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-IKKα (Ser180)/IKKβ (Ser181), and IKKα/β. 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 2.5×10^5 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-α/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

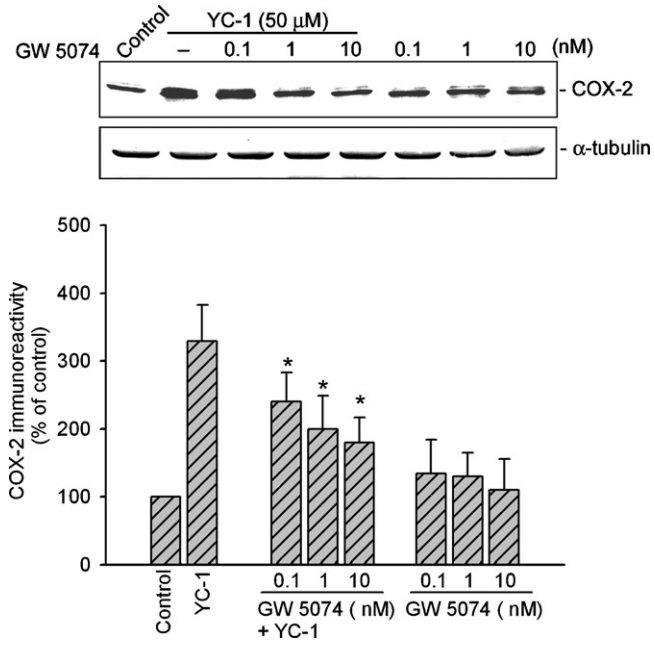

Fig. 1. Involvement of Raf-1 in YC-1-induced COX-2 expression in A549 cells. A549 cells were pretreated with various concentrations of GW 5074 for 30 min followed by incubation with 50 μM YC-1 for 12 h. Immunodetection with COX-2- or α-tubulin-specific antibodies was performed as described in Section 2. Equal loading in each lane was demonstrated by the similar intensities of α-tubulin. The extents of COX-2 and α-tubulin protein expressions were quantitated using a densitometer with Image-Pro plus software. The relative level was calculated as the ratio of COX-2 to α-tubulin protein levels. Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$, compared to the YC-1-treated group.

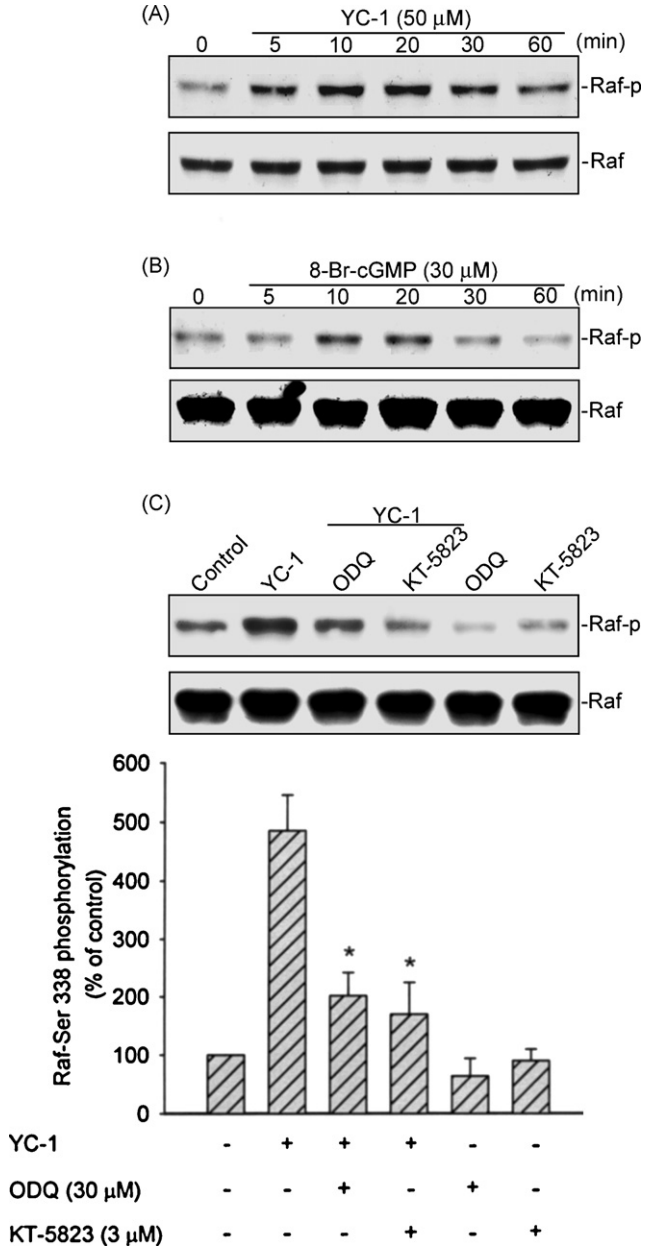

Fig. 2. Involvement of the sGC/cGMP/PKG pathway in YC-1-induced Raf-1 activation in A549 cells. Cells were treated with 50 μM YC-1 (A) or 30 μM 8-bromo-cGMP (B) for various time intervals. (C) Cells were pretreated with 30 μM ODQ or 3 μM KT-5823 for 30 min before incubation with 50 μM YC-1 for 20 min. 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.

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. Manunycin 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

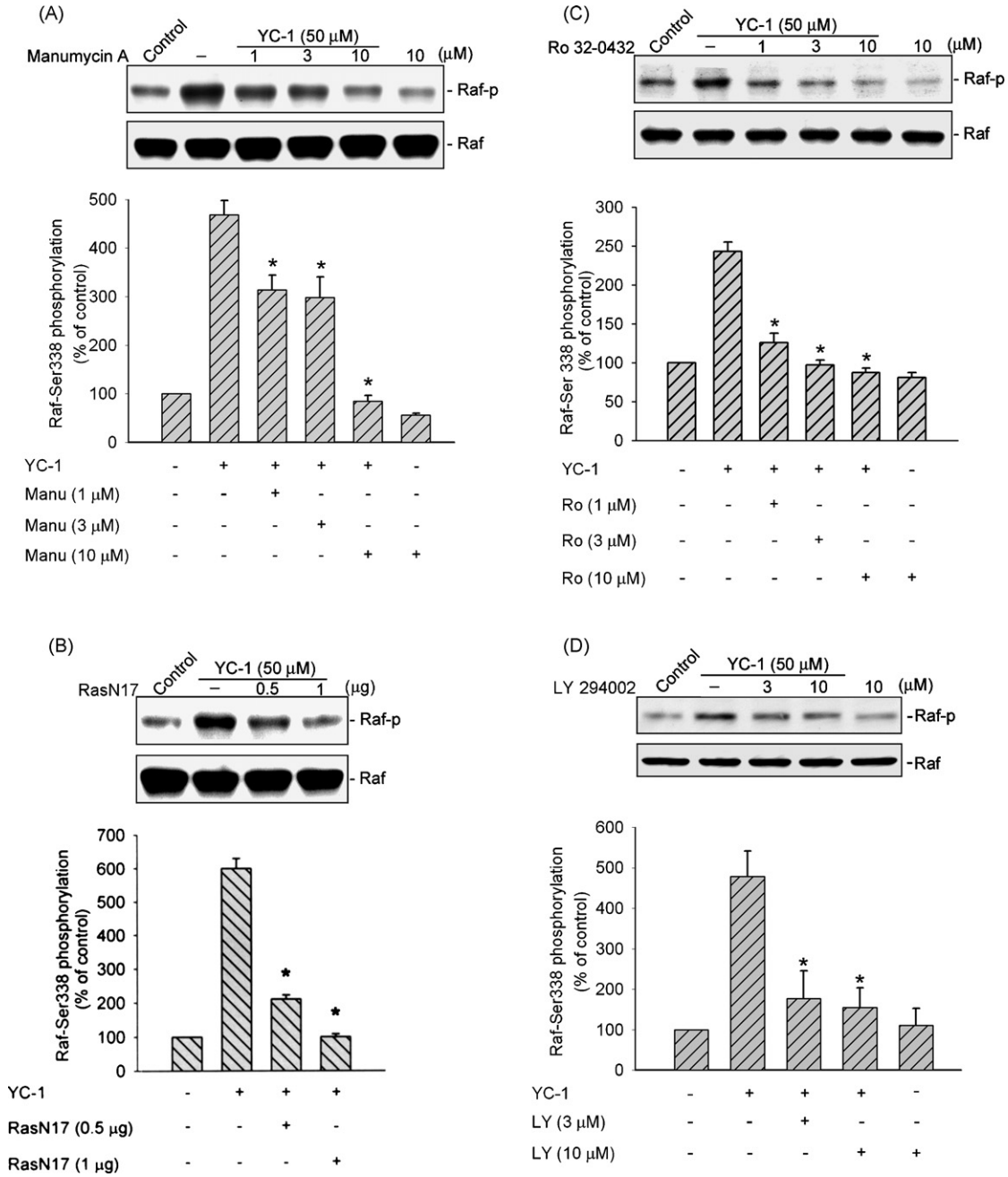

Fig. 3. Involvement of Ras, PKC-α, but not PI3K in YC-1-induced Raf-1 activation in A549 cells. Cells were pretreated with various concentrations of manumycin A (Manu) for 24 h (A), Ro 32-0432 (Ro) or LY 294002 (LY) for 30 min (C) and (D), or transfected with a dominant negative Ras mutant (RasN17) for 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).

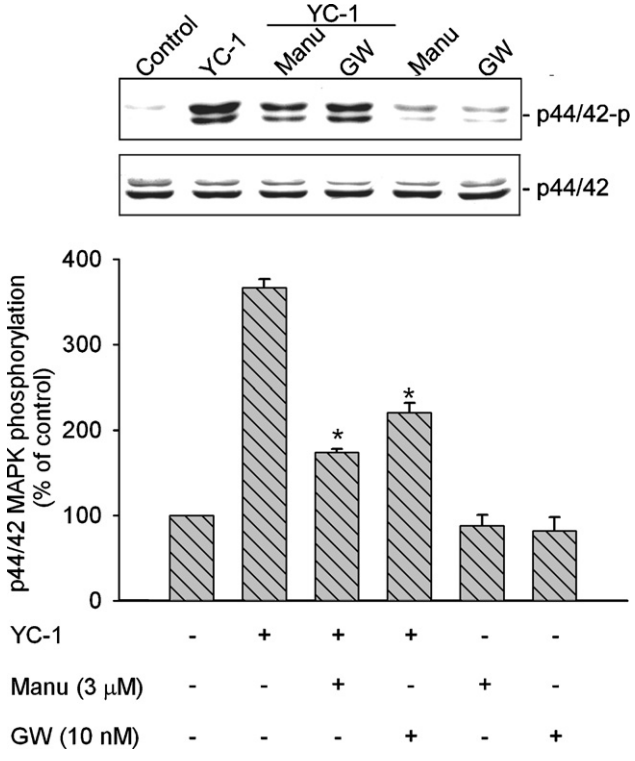

Fig. 4. Effects of manumycin A (Manu) and GW 5074 (GW) on YC-1-induced p44/42 MAPK activation in A549 cells. Cells were pretreated with 3 μM manumycin A for 24 h or 10 nM GW 5074 for 30 min before incubation with 50 μM YC-1 for 30 min. Cells were then prepared and subjected to Western blot analysis using 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 identical results. Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$, compared to the YC-1-treated group.

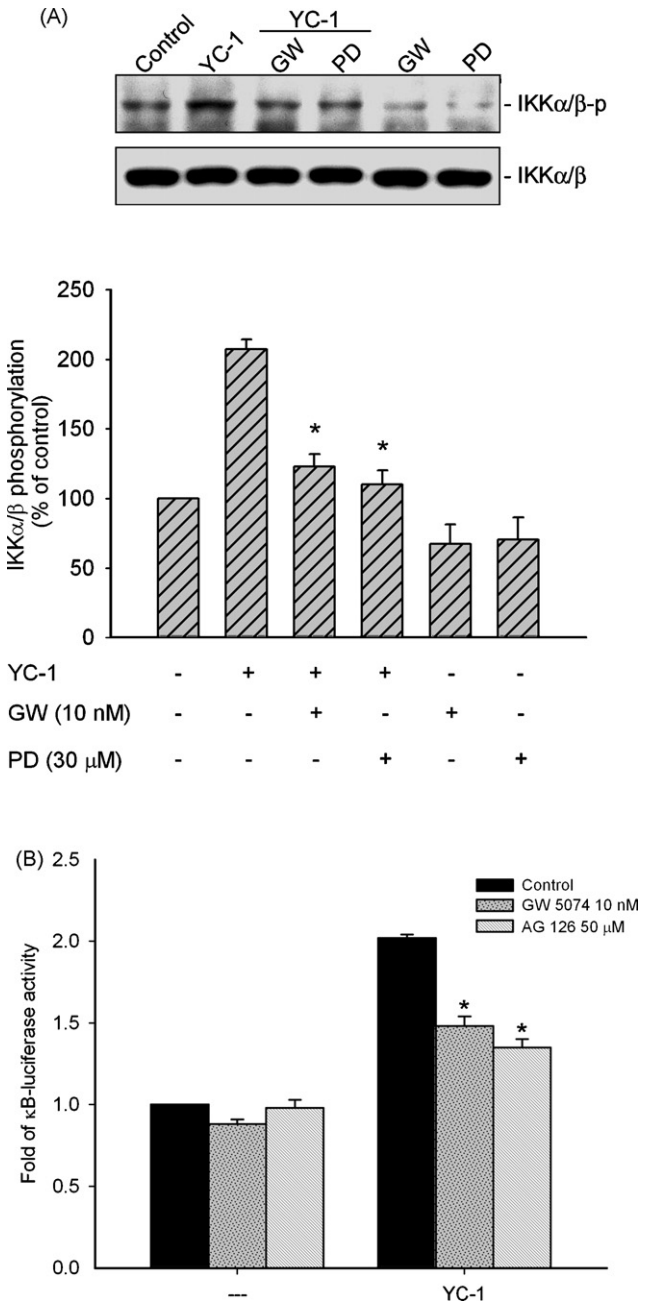

Fig. 5. Effects of various inhibitors on YC-1-induced increases in IKKα/β activation and κB-luciferase activity in A549 cells. (A) Cells were pretreated with 10 nM GW 5074 (GW) or 30 μM PD 98059 (PD) for 30 min before incubation with 50 μM YC-1 for 10 min. Cell lysates were prepared and subjected to 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 κB-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.

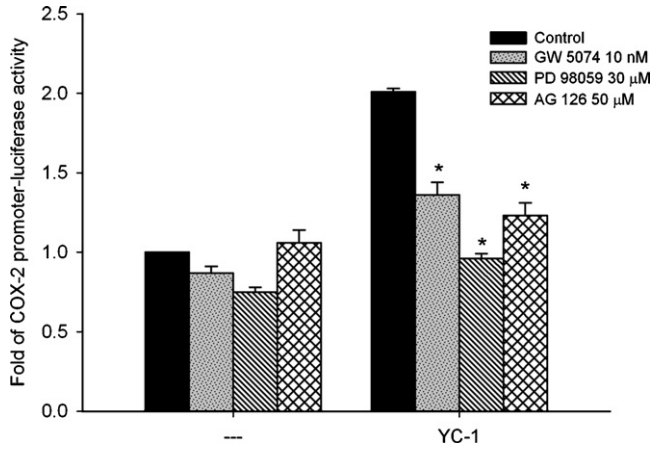

Fig. 6. Effects of various inhibitors on YC-1-induced COX-2 promoter-luciferase activity in A549 cells. Cells were transiently transfected with 1 μg of pGS459 (−459/+9) and 1 μg of pBK-CMV-Lac Z for 24 h, and then pretreated with 10 nM GW 5074, 30 μM PD 98059, or 50 μM AG 126 for 30 min, before incubation 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. ($n = 3$). *$p < 0.05$, compared to the YC-1-treated group.

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, pGS459 (−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 CL3 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 IκBα mutant (IκBαM), 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.

Acknowledgements

We are greatly indebted to Prof. Wan-Wan Lin for providing the plasmids, pGL2-ELAM-Luc and pBK-CMV-Lac Z. We also thank Prof. Che-Ming Teng and Prof. C.-C. Chen for providing plasmids RasN17 and pGS459, respectively.

References

[1] Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 1996;271:33157–60.
[2] Mitchell JA, Larkin S, Williams TJ. Cyclooxygenase-2: regulation and relevance in inflammation. Biochem Pharmacol 1995;50:1535–42.
[3] Portanova JP, Zhang Y, Anderson GD, Hauser SD, Masferrer JL, Seibert K, et al. Selective neutralization of prostaglandin E_2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. J Exp Med 1996;184:883–91.
[4] Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998;38:97–120.
[5] Vane JR, Mitchell JA, Appleton I, Tomlinson A, Bishop-Bailey D, Croxtall J, et al. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. Proc Natl Acad Sci USA 1994;91:2046–50.
[6] Kujubu DA, Fletcher BS, Varnum BC, Lim RW, Herschman HR. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem 1991;266:12866–72.
[7] Mitchell JA, Belvisi MG, Akarasereenont P, Robbins RA, Kwon OJ, Croxtall J, et al. Induction of cyclo-oxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone. Br J Pharmacol 1994;113: 1008–14.
[8] Devaliaand JL, Davies RJ. Airway epithelial cells and mediators of inflammation. Respir Med 1993;87:405–8.
[9] Campbell SL, Khosravi-Far R, Rossman KL, Clark GJ, Der CJ. Increasing complexity of Ras signaling. Oncogene 1998;17:1395–413.
[10] Zhang XF, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall MS, et al. Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 1993;364:308–13.
[11] Whitmarshand AJ, Davis RJ. Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. Trends Biochem Sci 1998;23:481–5.
[12] Kerkhoffand E, Rapp UR. The Ras–Raf relationship: an unfinished puzzle. Adv Enzyme Regul 2001;41:261–7.
[13] Luo Z, Diaz B, Marshall MS, Avruch J. An intact Raf zinc finger is required for optimal binding to processed Ras and for ras-dependent Raf activation in situ. Mol Cell Biol 1997;17:46–53.
[14] Williams JG, Drugan JK, Yi GS, Clark GJ, Der CJ, Campbell SL. Elucidation of binding determinants and functional consequences of Ras/Raf-cysteine-rich domain interactions. J Biol Chem 2000;275:22172–9.
[15] Subbaramaiah K, Hart JC, Norton L, Dannenberg AJ. Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2. Evidence for involvement of ERK1/2 AND p38 mitogen-activated protein kinase pathways. J Biol Chem 2000;275:14838–45.
[16] Van Putten V, Refaat Z, Dessev C, Blaine S, Wick M, Butterfield L, et al. Induction of cytosolic phospholipase A2 by oncogenic Ras is mediated through the JNK and ERK pathways in rat epithelial cells. J Biol Chem 2001;276:1226–32.
[17] Chen BC, Chang YS, Kang JC, Hsu MJ, Sheu JR, Chen TL, et al. Peptidoglycan induces nuclear factor-κB activation and cyclooxygenase-2 expression via Ras, Raf-1, and ERK in RAW 264.7 macrophages. J Biol Chem 2004;279:20889–97.
[18] Norrisand JL, Baldwin Jr AS. Oncogenic Ras enhances NF-κB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways. J Biol Chem 1999;274:13841–6.
[19] Baumann B, Weber CK, Troppmair J, Whiteside S, Israel A, Rapp UR, et al. Raf induces NF-κB by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. Proc Natl Acad Sci USA 2000;97:4615–20.
[20] Ko FN, Wu CC, Kuo SC, Lee FY, Teng CM. YC-1, a novel activator of platelet guanylate cyclase. Blood 1994;84:4226–33.
[21] Hwang TL, Wu CC, Teng CM. YC-1 potentiates nitric oxide-induced relaxation in guinea-pig trachea. Br J Pharmacol 1999;128:577–84.
[22] Tulis DA, Bohl Masters KS, Lipke EA, Schiesser RL, Evans AJ, Peyton KJ, et al. YC-1-mediated vascular protection through inhibition of smooth muscle cell proliferation and platelet function. Biochem Biophys Res Commun 2002;291:1014–21.
[23] Friebe A, Mullershausen F, Smolenski A, Walter U, Schultz G, Koesling D. YC-1 potentiates nitric oxide- and carbon monoxide-induced cyclic GMP effects in human platelets. Mol Pharmacol 1998;54:962–7.
[24] Chang MS, Lee WS, Teng CM, Lee HM, Sheu JR, Hsiao G, et al. YC-1 increases cyclo-oxygenase-2 expression through protein kinase G- and p44/42 mitogen-activated protein kinase-dependent pathways in A549 cells. Br J Pharmacol 2002;136:558–67.
[25] Chang MS, Lee WS, Chen BC, Sheu JR, Lin CH. YC-1-induced cyclooxygenase-2 expression is mediated by cGMP-dependent activations of Ras, phosphoinositide-3-OH-kinase, Akt, and nuclear factor-κB in human pulmonary epithelial cells. Mol Pharmacol 2004;66:561–71.
[26] Lin CH, Kuan IH, Wang CH, Lee HM, Lee S, Sheu JR, et al. Lipoteichoic acid-induced cyclooxygenase-2 expression requires activations of p44/42 and p38 mitogen-activated protein kinase signal pathways. Eur J Pharmacol 2002;450:1–9.
[27] Lackey K, Cory M, Davis R, Frye SV, Harris PA, Hunter RN, et al. The discovery of potent cRaf1 kinase inhibitors. Bioorg Med Chem Lett 2000;10:223–6.
[28] Diaz B, Barnard D, Filson A, MacDonald S, King A, Marshall M. Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. Mol Cell Biol 1997;17:4509–16.
[29] Chang MS, Chen BC, Yu MT, Sheu JR, Chen TF, Lin CH. Phorbol 12-myristate 13-acetate upregulates cyclooxygenase-2 expression in human pulmonary epithelial cells via Ras, Raf-1, ERK, and NF-κB, but not p38 MAPK, pathways. Cell Signal 2005;17:299–310.
[30] Wang CY, Wang YT, Tzeng DW, Yang JL. Lead acetate induces EGFR activation upstream of SFK and PKCα linkage to the Ras/Raf-1/ERK signaling. Toxicol Appl Pharmacol 2009;1:244–52.
[31] Duca L, Lambert E, Debret R, Rothhut B, Blanchevoye C, Delacoux F, et al. Elastin peptides activate extracellular signal-regulated kinase 1/2 via a Ras-independent mechanism requiring both p110γ/Raf-1 and protein kinase A/B-Raf signaling in human skin fibroblasts. Mol Pharmacol 2005;67:1315–24.
[32] Ghosh S, May MJ, Kopp EB. NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol 1998;16:225–60.
[33] DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature 1997;388:548–54.
[34] Arsura M, Mercurio F, Oliver AL, Thorgeirsson SS, Sonenshein GE. Role of the IkappaB kinase complex in oncogenic Ras- and Raf-mediated transformation of rat liver epithelial cells. Mol Cell Biol 2000;20:5381–91.