

PI-3K and Akt are mediators of AP-1 induction by 5-MCDE in mouse epidermal Cl41 cells

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5-Methylchrysene has been found to be a complete carcinogen in laboratory animals. However, the tumor promotion effects of (±)-anti-5-methylchrysene-1,2-diol-3,4-epoxide (5-MCDE) remain unclear. In the present work, we found that 5-MCDE induced marked activator protein-1 (AP-1) activation in Cl41 cells. 5-MCDE also induced a marked activation of phosphatidylinositol 3-kinase (PI-3K). Inhibition of PI-3K impaired 5-MCDE-induced AP-1 transactivation, suggesting that PI-3K is an upstream kinase involved in AP-1 activation by 5-MCDE. Furthermore, we found that Akt is a PI-3K downstream mediator for 5-MCDE-

induced AP-1 transactivation, whereas another PI-3K downstream kinase, p70^{S6K}, was not involved in AP-1 activation by 5-MCDE. Moreover, inhibition of Akt activation blocked 5-MCDE-induced activation of extracellular signal-regulated protein kinases (ERKs) and c-Jun NH₂-terminal kinases (JNKs), whereas it did not affect p38K activation. Consistently, overexpression of a dominant-negative mutant of ERK2 or JNK1 blocked the AP-1 activation by 5-MCDE. These results demonstrate that 5-MCDE is able to induce AP-1 activation, and the AP-1 induction is specifically through a PI-3K/Akt-dependent and p70^{S6K}-independent pathway.

Introduction

Polycyclic aromatic hydrocarbon (PAH) pollution is an ongoing leading environmental problem (Bostrom et al., 2002). PAHs are formed from the incomplete combustion of organic matter, such as coal, tobacco, petro fuels, wood, and municipal waste (International Agency for Research on Cancer, 1983). The carcinogenicity of PAHs has been clearly shown in both epidemiological studies and bioassays with laboratory animals (International Agency for Research on Cancer, 1983). The metabolite (±)-anti-5-methylchrysene-1,2-diol-3,4-epoxide (5-MCDE), one important member of PAH, has been found to be strongly carcinogenic (Hecht et al., 1974; Amin et al., 1991; You et al., 1994; Nesnow et al., 1998). Similar to (±)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide (B[a]PDE), 5-MCDE is the *in vivo* epoxide metabolite of its parent compound 5-methylchrysene, which has been identified in tobacco and marijuana smoke as well as gasoline and diesel engine exhaust (Hecht et al., 1974; Lee et al.,

1976; Grimmer, 1977). Chronic applications of 5-methylchrysene caused high incidences of lung cancer and skin cancer in several animal models (Hecht et al., 1974; Amin et al., 1991; You et al., 1994; Nesnow et al., 1998). Most of the previous works have focused on the mutagenicity of 5-methylchrysene and found that its metabolites, especially 5-MCDE, bind covalently to the N2-guanine base in the DNA and subsequently cause mutation (Melikian et al., 1984; Amin et al., 1985; Hecht et al., 1987; Reardon et al., 1987). To substantiate that 5-MCDE exposure is one of the causative factors of lung cancer associated with cigarette smoking, Smith et al. (2000) have demonstrated that the G to T mutation spectrum caused by 5-MCDE in the p53 tumor suppressor gene perfectly matches the mutation hotspots found in lung cancer patients who are cigarette smokers. These mutagenicity studies on 5-MCDE convincingly explain the tumor initiator effects of 5-MCDE. From animal model assays for carcinogenicity, it is well accepted

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Abbreviations used in this paper: 5-MCDE, (±)-anti-5-methylchrysene-1,2-diol-3,4-epoxide; AP-1, activator protein-1; B[a]P, benzo[a]pyrene; B[a]PDE, (±)-anti-benzo[a]pyrene-7,8-diol-9,10-epoxide; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun NH₂-terminal kinase; PAH, polycyclic aromatic hydrocarbon; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

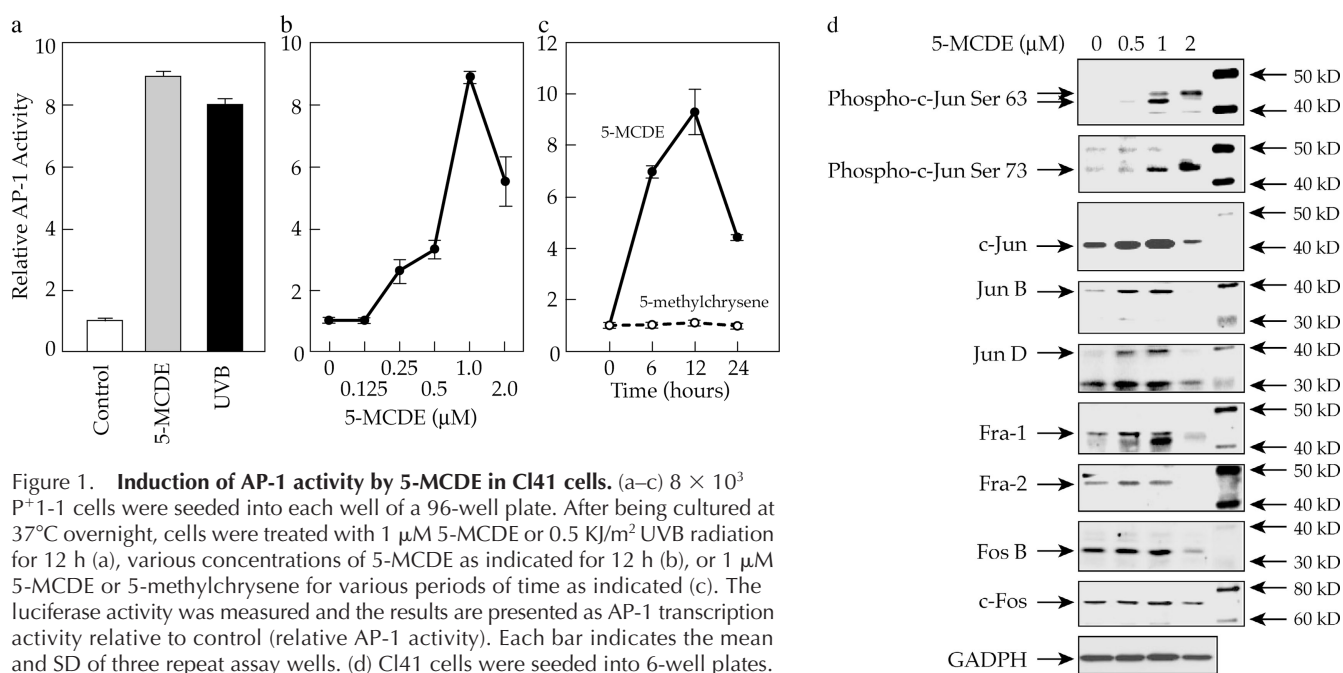


Figure 1. Induction of AP-1 activity by 5-MCDE in C141 cells. (a–c) 8×10^3 P⁺1-1 cells were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, cells were treated with 1 μM 5-MCDE or 0.5 KJ/m² UVB radiation for 12 h (a), various concentrations of 5-MCDE as indicated for 12 h (b), or 1 μM 5-MCDE or 5-methylchrysene for various periods of time as indicated (c). The luciferase activity was measured and the results are presented as AP-1 transcription activity relative to control (relative AP-1 activity). Each bar indicates the mean and SD of three repeat assay wells. (d) C141 cells were seeded into 6-well plates. The media were replaced with 0.1% FBS MEM after the cell density reached 70–80% confluency. 45 h later, the cells were incubated with fresh serum-free MEM for 3–4 h at 37°C. Cells were exposed to 1 μM 5-MCDE for 420 min. The cells were washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide–SDS gels, transferred, and probed with one of the specific antibodies as indicated. The protein band specific bound with primary antibodies was detected by using anti-rabbit IgG-AP-linked and ECF Western blotting system.

that the mutated cell needs to undergo tumor promotion and progression to develop into a fully-grown tumor and, ultimately, cancer. Because 5-methylchrysene was shown to be a complete carcinogen in the animal models (Hecht et al., 1974; Amin et al., 1991; You et al., 1994; Nesnow et al., 1998), it is interesting and practically important to know whether or not 5-methylchrysene and/or better certain of its metabolites also function as tumor-promoting agents. However, to our knowledge, the mechanisms of tumor promotion effects of 5-methylchrysene and its metabolites have been barely understood.

The tumor promotion phase is a long-term reversible process characterized as the activation of transcription factors and regulation of their target genes through signal transduction pathways (Slaga and Fischer, 1983; Pitot and Dragan, 1996). The mouse epidermal JB6 C141 cell line is a well-characterized and widely used cell culture model for tumor promotion studies (Bernstein and Colburn, 1989; Dong et al., 1994, 1995; Huang et al., 1997a, 1998; Li et al., 1997; Watts et al., 1998). In transformation-sensitive (P⁺) but not transformation-resistant (P[−]) JB6 cell lines, tumor promoters, such as phorbol esters or growth factors, induce activator protein-1 (AP-1) activity and neoplastic transformation (Bernstein and Colburn, 1989). Inhibition of AP-1 induction by TAM67 (a transactivation domain deletion mutant of c-Jun; Brown et al., 1993) blocks 12-*O*-tetradecanoylphorbol-13-acetate (TPA)- and EGF-induced AP-1 transactivation and cell transformation (Dong et al., 1997). Several other events are also found to be required for different chemical-induced tumor promotion, including activation of extracellular signal-regulated protein kinases (ERKs), nuclear factor-κB, and PI-3K (Huang et al., 1996, 1997b, 1998; Li et al., 1997; Watts

et al., 1998; Hsu et al., 2000). Importantly, this cell line is derived from mouse skin; thus, the results generated from C141 cells will provide a strong basis for further investigation in mouse skin, which is an important bioassay system for the evaluation of tumor promotion in vivo (Slaga and Fischer, 1983; Pitot and Dragan, 1996). Therefore, C141 cells were used in this paper as the in vitro model to investigate the tumor-promoting activity of 5-MCDE, the metabolite of 5-methylchrysene. We found that 5-MCDE induces AP-1 transcriptional activity in the C141 cell line, and this induction occurs via a PI-3K/Akt-dependent signaling pathway.

Results

Induction of transactivation of AP-1 in C141 cells by 5-MCDE

AP-1 is the transcription factor demonstrated to be required for cancer development by chemical carcinogens. AP-1 is composed of a heterogeneous set of dimeric proteins, including the members from the Jun, Fos, and ATF families (Karin et al., 1997). Whereas Jun is expressed constitutively but activated after phosphorylation, the Fos is activated by regulation of its protein expression (Karin et al., 1997). It has been reported that PAHs and/or their metabolites, such as benzo[a]pyrene (B[a]P), affect cellular signaling pathways (Parrish et al., 1998). Therefore, we first looked at the change of AP-1 transcriptional activity after 5-MCDE treatment in the P⁺1-1 cells, which are the C141 cell line stably transfected with AP-1 luciferase reporter. After treating P⁺1-1 cells with noncytotoxic dose of 5-MCDE, AP-1 transcriptional activity was induced at the level similar to UVB radiation, which is a well-known oxidative stress and skin carcinogenic factor (Fig. 1 a).

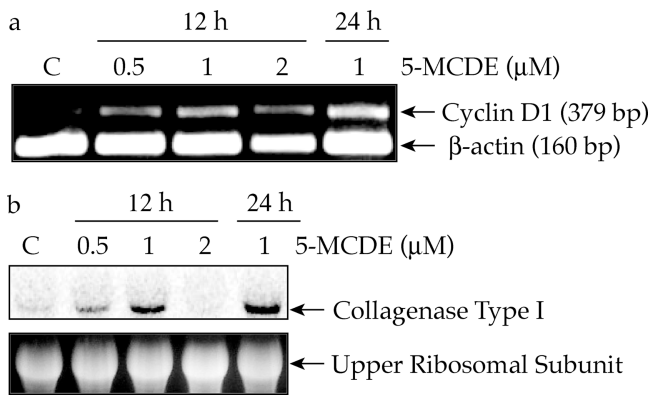


Figure 2. 5-MCDE induces the transcription of AP-1 target genes. C141 cells were seeded into 100-mm dishes. After being cultured at 37°C overnight, cells were treated with various concentrations of 5-MCDE as indicated for 12 or 24 h. RNA was isolated as described in Materials and methods. (a) RT-PCR for cyclin D1 and β -actin were performed as described in Materials and methods. (b) Total RNA (15 μ g) was subjected to Northern blot analysis and hybridized to mouse *MMP13* probe. The ethidium bromide staining of the upper ribosomal subunit is the control to ensure equal RNA loading.

5-MCDE-induced AP-1 transactivation appeared in a dose- and time-dependent manner (Fig. 1, b and c). In contrast with the AP-1 activation by 5-MCDE, 5-methylchrysene did not show any inductive effect on AP-1 activity in C141 cells (Fig. 1 c). Further studies showed that at 7 h after treatment, 5-MCDE did increase in expression of AP-1 family members, including c-Jun, Jun B, Jun D, Fra-1, Fra-2, and Fos B (Fig. 1 d), whereas it did not affect these protein's expression at 3 h after treatment (not depicted). In contrast with these inductions, 5-MCDE did not induce c-Fos protein expression at all the time points tested (Fig. 1 d and not depicted). More importantly, c-Jun phosphorylations at serine63 and serine73 were markedly induced by 5-MCDE treatment (Fig. 1 d). To test whether or not 5-MCDE is able to induce AP-1 target gene expression, we evaluated the effects of 5-MCDE on expression of a few AP-1 target genes, such as collagenase type I (*MMP13*) and cyclin D1. As shown in Fig. 2, AP-1 target genes, cyclin D1 and collagenase type I, were significantly induced by 5-MCDE exposure. These results indicate that 5-MCDE, a major metabolite of 5-methylchrysene, is able to induce AP-1 activation and AP-1 target gene expression.

Induction of PI-3K activity in C141 cells by 5-MCDE

AP-1 activity may be regulated through various signaling pathways. Previous works have indicated that PI-3K activation is required for AP-1 activation by EGF (Huang et al., 1996), TPA (Huang et al., 1997b), or IL-1 (Sizemore et al., 1999). In contrast, some other works showed that PI-3K is not involved in AP-1 activation by lipopolysaccharide, oncogenic 70Z Cb1, and activated MEK, even though PI-3K is activated by lipopolysaccharide or MEK (Treinies et al., 1999; van Leeuwen et al., 1999; Guha and Mackman, 2002). Thus, AP-1 activation could be activated via PI-3K-dependent or -independent pathways. To elucidate the upstream signals responsible for induction of AP-1 activity by 5-MCDE, the activity of PI-3K was measured in C141 cells. As shown in Fig. 3, treatment of cells with 5-MCDE led to marked PI-3K acti-

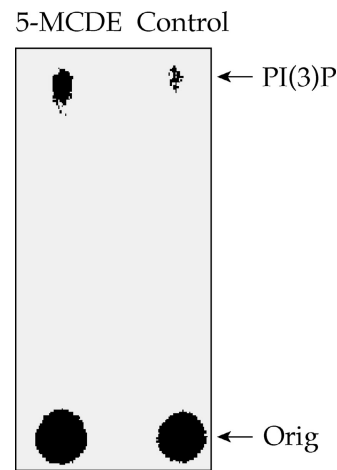


Figure 3. Induction of PI-3K activity by 5-MCDE in C141 cells. C141 cells were seeded into each of 100-mm dishes. The media were replaced with 0.1% FBS MEM after the cell density reached 70–80% confluency. 45 h later, the cells were incubated with fresh serum-free MEM for 3–4 h at 37°C. Cells were exposed to 1 μ M 5-MCDE for 20 min. Cells were washed once with ice-cold PBS and lysed in 400 μ l of lysis buffer. PI-3K activity was measured as described in Materials and methods.

vation at 20 min after exposure. This result reveals that PI-3K is activated in C141 cells in response to 5-MCDE exposure.

PI-3K activation is required for AP-1 transactivation by 5-MCDE in C141 cells

Because the aforementioned results suggested that both AP-1 and PI-3K are activated in C141 cells in response to 5-MCDE exposure, it was of interest to determine the feasible role of PI-3K in 5-MCDE-stimulated AP-1 transactivation in C141 cells. We first assessed the effects of two PI-3K chemical inhibitors, wortmannin and Ly294002, on 5-MCDE-induced AP-1 activation. The results showed that unsaturated doses of both wortmannin and Ly294002 did reduce the AP-1 activation by 5-MCDE (Fig. 4 a). To further prove the role of PI-3K in 5-MCDE-induced AP-1 activation, C141 AP-1 Δ p85 mass1, which is a stable transfectant of a dominant-negative mutant p85 subunit of PI-3K (Δ p85), was used (Huang et al., 1996, 1997b). An overexpression of Δ p85 almost completely impaired 5-MCDE-induced AP-1 activation (Fig. 4, b–d). This inhibition was present at all the time points and doses tested in C141 AP-1 Δ p85 mass1 cell (Fig. 4, c and d). All these results clearly show that 5-MCDE induces AP-1 activation through a PI-3K-mediated signaling pathway.

Overexpression of dominant-negative mutant Akt blocked 5-MCDE-induced AP-1 activation in C141 cells

The primary consequence of PI-3K activation is the phosphorylation of PtdIns(4,5)P₂ (PIP₂) into PtdIns(3,4,5)P₃ (PIP₃), which acts as a second messenger. PIP₃ recruits Akt, a serine/threonine kinase, to the plasma membrane by direct interaction with its PH domain (Andjelkovic et al., 1997; Bellacosa et al., 1998). At the membrane, Akt can be phosphorylated at Serine 473 and Threonine 308, and thus be activated to exhibit its biological effect (Vanhaesebroeck and Alessi, 2000). From our previous work, it is known that Akt is one of PI-3K

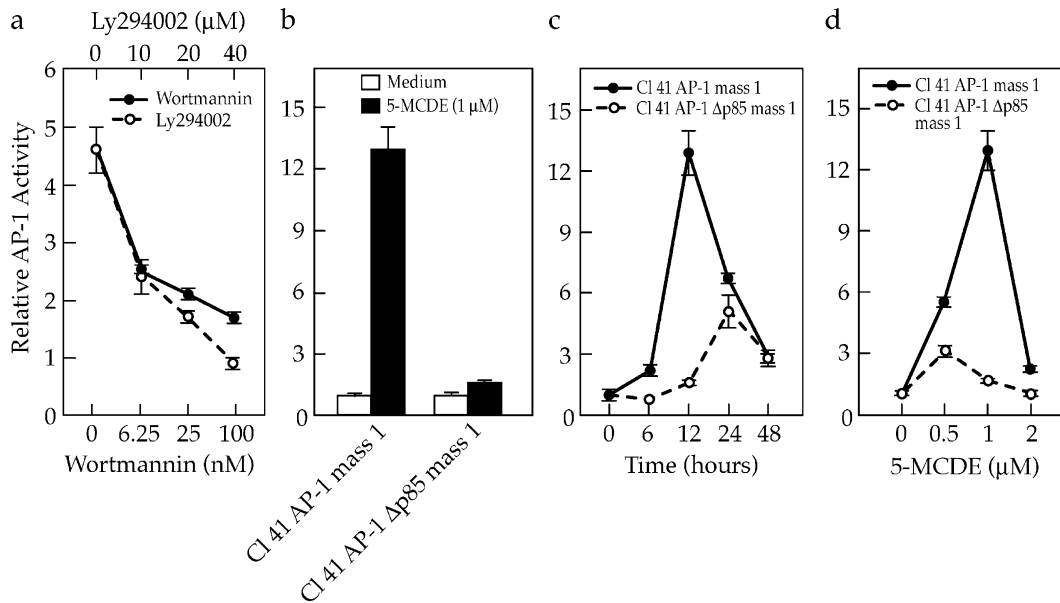
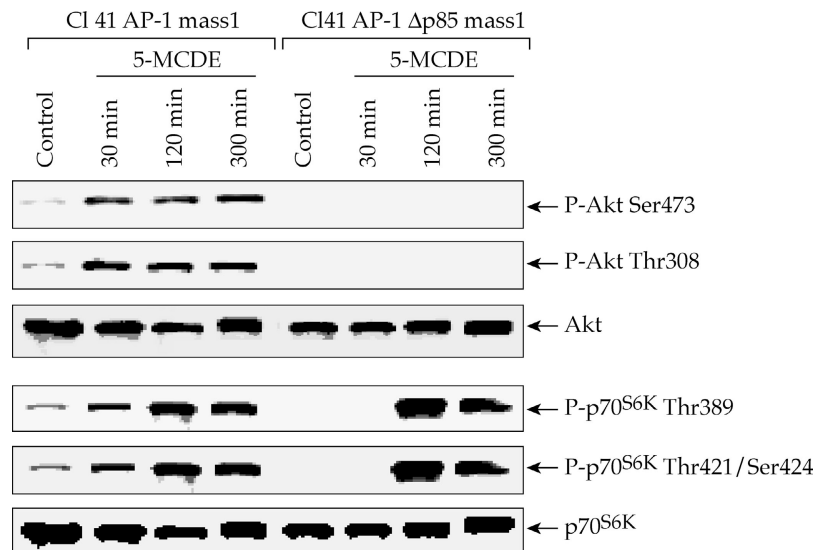


Figure 4. Reduction of 5-MCDE-induced AP-1 activity by inhibition of PI-3K activation. 8×10^3 P⁺1-1, Cl41 AP-1 mass1, or Cl41 AP-1 Δp85 mass1 were seeded into each well of 96-well plates. After being cultured at 37°C overnight, P⁺1-1 cells were pretreated with various concentrations of wortmannin or Ly294002 as indicated for 30 min (a), and then exposed to 1 μM 5-MCDE for 12 h. (b–d) Cl41 AP-1 mass1 or Cl41 AP-1 Δp85 mass1 cells were treated with 1 μM 5-MCDE for 12 h (b), 1 μM 5-MCDE for various time points as indicated (c), or various concentrations of 5-MCDE for 12 h (d). The luciferase activity was measured and the results are presented as relative AP-1 activity. Each bar indicates the mean and SD of four repeat assay wells.

downstream kinases in Cl41 cells in response to UV radiation (Huang et al., 2001). However, it is not clear whether or not Akt is activated by 5-MCDE. Also, there is no evidence to show if Akt is required for AP-1 activation. To determine the potential involvement of Akt in 5-MCDE-induced AP-1 activation, we first compared the phosphorylation levels of Akt at Thr308 and Ser473 in Cl41 AP-1 Δp85 mass1 with those in Cl41 AP-1 mass1 after 5-MCDE exposure. In Cl41 AP-1 mass1 cells, there were obvious increases of phosphorylations at Akt Ser473 and Thr308 at different time points after 5-MCDE exposure, whereas in Cl41 AP-1 Δp85 mass1 cells, which express dominant-negative p85, the phosphorylations at Akt Ser473 and Thr308 were completely impaired (Fig. 5).

Together with the data on AP-1 induction after 5-MCDE exposure in Cl41 AP-1 Δp85 mass1 cells, it suggested that PI-3K might induce AP-1 transactivity through Akt kinase. To further explore the role of Akt in 5-MCDE-induced AP-1 activation, we established a stable transfectant with the dominant-negative mutant Akt, Cl41 AP-1 Akt-T308A/S473A mass1. In contrast to the induction of phosphorylations of Akt at Thr308 and Ser473 after 5-MCDE in the Cl41 AP-1 mass1 cell, the expression of dominant-negative Akt-T308A/S473A specifically blocked the increases of phosphorylations of Akt at Thr308 and Ser473 (Fig. 6 a). Consistent with Akt activation, in Cl41 DN Akt-T308A/S473A mass1 cells, c-Jun phosphorylation and AP-1 activation were inhibited com-

Figure 5. Effects of Δp85 overexpression on phosphorylation of Akt and p70^{S6K} induced by 5-MCDE. Subconfluent (90%) monolayers of Cl41 AP-1 mass1 and Cl41 AP-1 Δp85 mass1 in 6-well plates were subjected to 1 μM 5-MCDE for various periods of time as indicated. The cells were washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with one of the specific antibodies as indicated. The protein band specific bound with primary antibodies was detected by using anti-rabbit IgG-AP-linked and ECF Western blotting system.



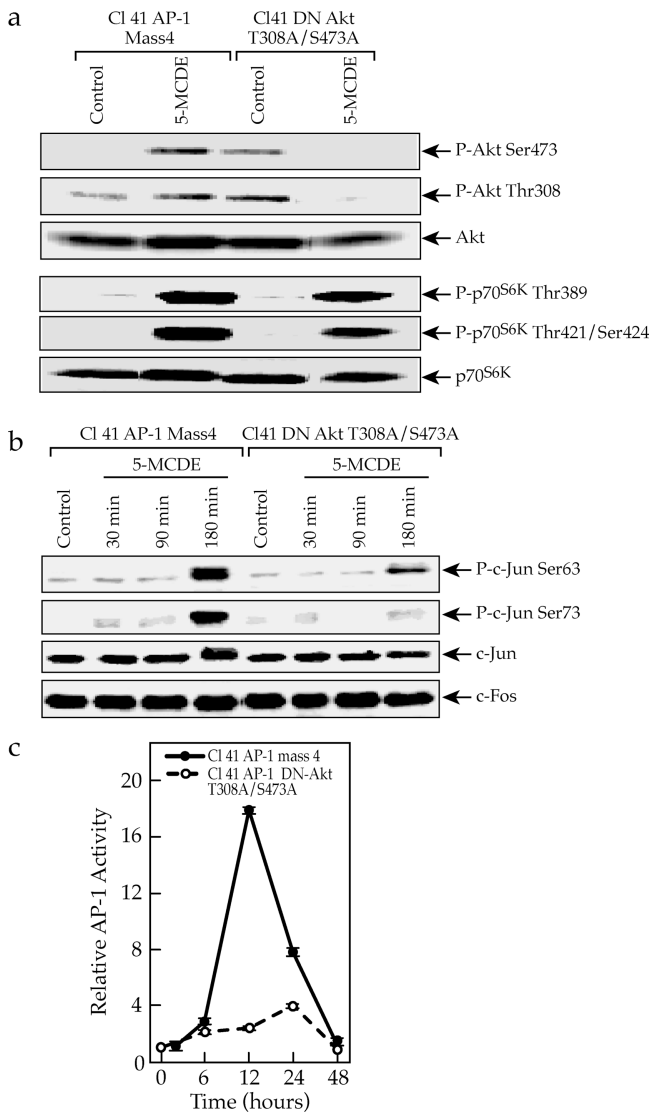


Figure 6. Effects of overexpression of AktT308A/S473A on 5-MCDE-induced AP-1 activity and c-Jun phosphorylation. (a and b) For assay of phosphorylation of Akt, p70^{S6K}, or c-Jun, subconfluent (90%) monolayers of CI41 AP-1 mass4 or CI41 AP-1 DN-Akt T308A/S473A in 6-well plates were subjected to 1 μ M 5-MCDE for 180 min (a) or time periods as indicated (b). The cells were extracted and Western blot was performed with specific antibodies as indicated. (c) 8×10^3 of CI41 AP-1 mass4 or CI41 AP-1 DN-Akt T308A/S473A were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were exposed to 1 μ M 5-MCDE for various time points as indicated. The luciferase activity was measured and the results are presented as relative AP-1 activity. Each bar indicates the mean and SD of four repeat assay wells.

pared with that in CI41 AP-1 mass4 cells (Fig. 6, b and c). These results demonstrate that Akt is the downstream kinase responsible for the activation of AP-1 by 5-MCDE.

p70^{S6K} is not involved in 5-MCDE-induced AP-1 transactivation

Another important serine/threonine kinase related to PI-3K and the Akt cascade is mTOR (the mammalian target of rapamycin, also known as FRAP1), which regulates biosynthesis by inhibiting 4E-BP1 and activating p70^{S6K} via phosphor-

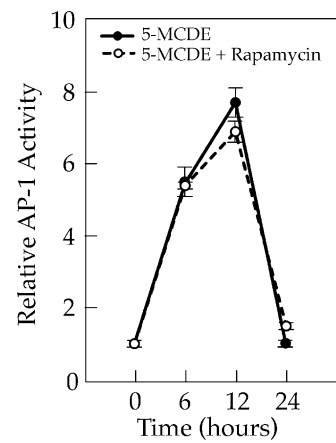


Figure 7. Inhibition of mTOR-p70^{S6K} pathway by rapamycin does not affect 5-MCDE-induced AP-1 transactivation. 8×10^3 P⁺1-1 cells were seeded into each well of a 96-well plate. After being cultured at 37°C overnight, cells were pretreated with 20 μ M rapamycin for 30 min and exposed to 1 μ M 5-MCDE for various time points as indicated. The luciferase activity was measured and the results are presented as relative AP-1 activity. Each bar indicates the mean and SD of four repeat assay wells.

ylation at its specific threonine and serine sites (Schmelzel and Hall, 2000). There are many evidences showing that mTOR is regulated directly by PI-3K (Brunn et al., 1996; Nave et al., 1999). To explore the role of the mTOR-p70^{S6K} cascade in AP-1 activation by 5-MCDE, we first measured the level of the phosphorylated form of p70^{S6K} in exposed cells. As shown in Fig. 5, there was an increase in phosphorylation of p70^{S6K} at Thr389 and Thr421/Ser424 in CI41 AP-1 mass1 cells after the exposure, whereas an introduction of dominant-negative mutant PI-3K (Δ p85) only inhibited 5-MCDE-induced phosphorylation of p70^{S6K} in the early phase (30 min after 5-MCDE exposure), but not at later time points (Fig. 5, 120 or 300 min after exposure). Furthermore, an overexpression of Akt-T308A/S473A did not show any inhibitory effect on phosphorylation of p70^{S6K} at Thr389 and Thr421/Ser424 (Fig. 6 a). This finding suggests that 5-MCDE is able to induce p70^{S6K} phosphorylation at Thr389 and Thr421/Ser424 through both PI-3K-dependent and -independent pathways, and Akt activation is not critical for p70^{S6K} activation in CI41 cells. Furthermore, it was found that inhibition of p70^{S6K} by pretreatment of cells with rapamycin, a specific inhibitor for mTOR, did not show any inhibitory effect on AP-1 transactivation induced by 5-MCDE at various time points (Fig. 7). The results indicate that 5-MCDE is able to activate p70^{S6K}, however, this activation is not required for AP-1 transactivation by 5-MCDE in mouse C141 cells.

Dominant-negative mutant Akt impaired 5-MCDE-induced AP-1 activation specifically through inhibition of activation of ERKs and c-Jun NH₂-terminal kinases (JNKs)

AP-1 has been identified as a target of the MAPKs family, including ERKs, JNKs, and p38K (Karin et al., 1997). To understand the interaction between MAPKs and PI-3K on the regulation of AP-1 transactivation, the effects of

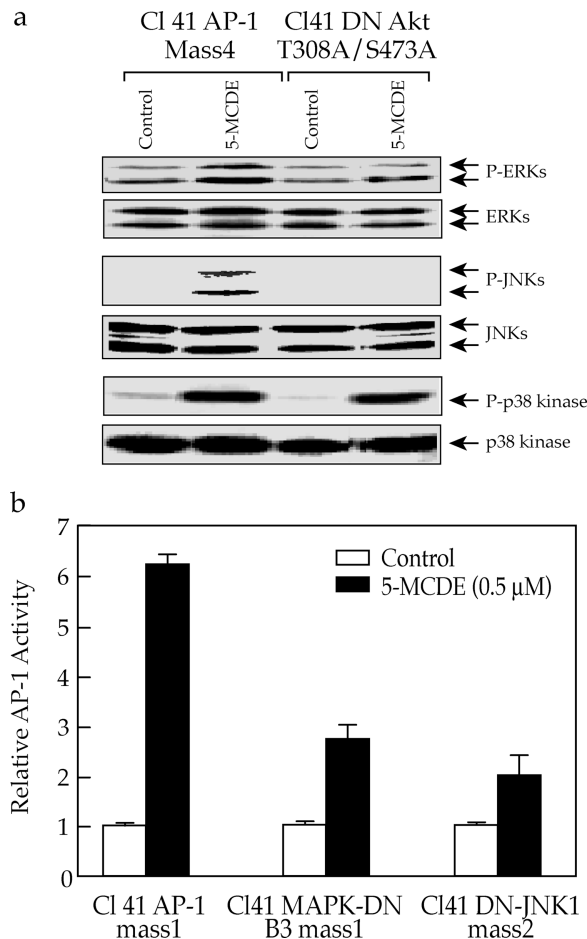


Figure 8. 5-MCDE induces AP-1 activation through Akt-ERK and JNK pathway. (a) Subconfluent (90%) monolayers of Cl41 AP-1 mass4 or Cl41 AP-1 DN-Akt T308A/S473A in 6-well plates were subjected to 1 μM 5-MCDE for 90 min. The cells were extracted and Western blot was performed as described in Materials and methods with specific antibodies as indicated. (b) 8×10^3 Cl41 AP-1 Mass1, Cl41 MAPK-DN B3 mass1, or Cl41 DN-JNK1 mass2 were seeded into each well of 96-well plates. After being cultured at 37°C overnight, cells were exposed to 0.5 μM 5-MCDE for 12 h. The luciferase activity was measured and the results are presented as relative AP-1 activity. Each bar indicates the mean and SD of triplicate assay wells.

5-MCDE on phosphorylations of the MAPKs were evaluated in the cell transfected with dominant-negative mutant Akt, Akt-T308A/S473A. Consistent with AP-1 induction, exposure of Cl41 cells to 5-MCDE led to an increase in the phosphorylations of all three members of MAPKs, the ERKs, JNKs, and p38K (Fig. 8 a). However, an overexpression of dominant-negative mutant Akt, Akt-T308A/S473A, specifically inhibited 5-MCDE-induced phosphorylations of ERKs and JNKs but not p38K (Fig. 8 a). To further investigate the role of ERKs and JNKs in 5-MCDE-induced AP-1 activation, well-characterized dominant-negative mutant ERK2 (Cl41 MAPK-DN B3 mass1) or JNK1 Cl41 cell stable transfectants (Huang et al., 1999a,b) were used. As shown in Fig. 8 b, overexpression of dominant-negative mutant ERK2 or JNK1 markedly inhibited AP-1 activation by 5-MCDE. All these data show that Akt mediates AP-1 activation by 5-MCDE specifically through ERKs and JNKs but not p38K.

Discussion

PAH contamination gets worldwide concerns for its impact on human health and living environment. Exposures to PAHs are associated with an increased risk of cancers in various organs and tissues, including lung and skin (Hecht et al., 1974; International Agency for Research on Cancer, 1983; Bernelot-Moens et al., 1990; Amin et al., 1991; You et al., 1994; Nesnow et al., 1998). 5-MCDE, the PAH studied in this paper, is an *in vivo* metabolite of 5-methylchrysene, which has been shown to be a complete carcinogen in laboratory animals (Hecht et al., 1974; Amin et al., 1991; You et al., 1994). Similar to the activation of B[a]P, 5-methylchrysene is metabolized into its active epoxide metabolite in the skin by CYP1A1 and CYP1B1 through the following pathway: 5-methylchrysene → 1,2-dihydroxy-5-methylchrysene → 5-MCDE (Shimada et al., 2001). Previous studies have focused on the mutagenicity of 5-MCDE, which is largely due to the reactivity of 5-MCDE with DNA. Different from the weak mutagenicity of the epoxide metabolites of other methylchrysene derivatives, 5-MCDE is a strong mutagen due to its methyl group locating in the bay region. Thus, it binds to the N2-guanosine site by a special stereo-structure, forming adducts similar to those derived from B[a]PDE (Melikian et al., 1984; Amin et al., 1985; Hecht et al., 1987; Reardon et al., 1987). These 5-MCDE-guanosine adducts cause subsequent mutations and may activate *k-ras* oncogene and/or inactivate tumor suppressor gene *p53* (You et al., 1994; Denissenko et al., 1996). In this paper, we present additional evidence that 5-MCDE may contribute to tumorigenesis by inducing AP-1 transactivation through PI-3K, Akt, MAPK, and JNK cascades, which have been found to be required for tumor promotion and progression.

AP-1 is an important transcription factor that governs the expression of genes involved in intercellular communication, amplification, and primary pathogenic signals spreading as well as initiation and acceleration of tumorigenesis (Shaulian and Karin, 2002). It recognizes both TPA response elements (5'-TGAG/CTCA-3') and cAMP response elements (5'-TGACGTCA-3'; Chinenov and Kerppola, 2001). The Cl41 cell line, which is used in this paper, proceeds to an anchorage-independent growth phenotype and tumorigenicity upon the induction by phobol ester (TPA), EGF, or TNF-α (Huang et al., 1996, 1997b, 1999a; Watts et al., 1998). AP-1 activation is identified as one of the required events for the aforementioned transformations (Colburn and Smith, 1987; Bernstein and Colburn, 1989; Dong et al., 1994; Singh et al., 1995; Huang et al., 1997a). Both the AP-1 molecular inhibitor TAM67 (a transactivation domain deletion mutant of c-Jun) and AP-1 transrepressing retinoids can block TPA-induced AP-1 transactivation, cell transformation, and tumor induction (Dong et al., 1994; Huang et al., 1997a,b). Thus, the importance of AP-1 in tumor promotion is well established. The mechanisms of cell transformation by AP-1 are probably through its manipulation on the target genes expressions (Chinenov and Kerppola, 2001; Shaulian and Karin, 2001). For instance, c-Jun is a positive regulator on gene expression of cyclin D1 and FasL, and a negative regulator of p16^{ink4a}, p53, and p21^{cip1/waf1} (Shaulian and Karin, 2001), whereas c-Fos is required for expression of many matrix metalloproteinases

(Saez et al., 1995). In this paper, 5-MCDE was shown to induce AP-1 transactivation. Although it does not change the c-Fos protein level, 5-MCDE exposure increases the expressions of Jun B, Fos B, Fra-1, and Fra-2. More importantly, the phosphorylations of c-Jun protein at serines 63 and 73 are markedly induced after 5-MCDE treatment. Furthermore, 5-MCDE exposure also increases the expression of AP-1 target genes, such as cyclin D1 and collagenase type I, which are associated with cell cycle regulation and tumor induction, respectively (Schonthal et al., 1988; Saez et al., 1995; Shaulian and Karin, 2002). Because 5-MCDE is identified as a complete carcinogen in animal carcinogenesis models, our results strongly suggest that AP-1 activation is one of the events necessary for the tumor promotion by 5-MCDE.

In the process of identifying the upstream-regulating signal transduction pathway, we found that the PI-3K pathway is involved in 5-MCDE-induced AP-1 activation. Using both PI-3K inhibitor (wortmannin and LY294002) and the dominant-negative mutant PI-3K ($\Delta p85$) can substantially inhibit AP-1 transactivation induced by 5-MCDE. This result is consistent with our previous findings that PI-3K activation is the upstream kinase event responsible for mediation of AP-1 transactivation induced by TPA or EGF (Huang et al., 1996, 1997b). PI-3K also plays an important role in skin tumor progression phase. It is suggested by the observations that wortmannin and dominant-negative p85 subunit of PI-3K inhibit the TPA- and EGF-induced cell transformation and prevent the invasion of MDA-MB-435 cells, whereas a constitutively active p110 subunit of PI-3K increases their invasion (Huang et al., 1996, 1997b; Adelman et al., 1999). Together, we speculate that the PI-3K signal pathway is involved in carcinogenic effects of 5-MCDE.

Among the downstream kinases of PI-3K, Akt has been identified as the main signal transmitter. Akt, which is also known as PKB, is the cellular homologue of the retroviral oncogene v-Akt (Datta et al., 1999; Scheid and Woodgett, 2001). The activation of Akt has been identified in many cancers (Cheng et al., 1996; Bellacosa et al., 1998; Ruggeri et al., 1998; Shayesteh et al., 1999). For instance, amplification of Akt was observed in cancers of the breast, ovaries, and pancreas (Cheng et al., 1996; Bellacosa et al., 1998; Ruggeri et al., 1998; Shayesteh et al., 1999). The loss of function of tumor suppressor gene PTEN, which converts PIP3 back to PIP2 and thus mitigates the effects of Akt activation caused by PI-3K, is also seen in a wide spectrum of human cancers (Ali et al., 1999). Besides the PI-3K-dependent pathway, increased calcium can also activate the calcium/calmodulin-dependent kinase kinase, which then activates Akt by directly phosphorylating Akt at the Thr³⁰⁸ (Chen et al., 2002). In this work, Akt was found to be involved in 5-MCDE-induced AP-1 transactivation and activated in a PI-3K-dependent manner. Overexpression of a dominant-negative subunit of PI-3K blocked the 5-MCDE-induced Akt phosphorylations at Ser473 and Thr308. Introduction of dominant negative mutant Akt specifically impairs 5-MCDE-induced activation of AP-1. These results indicate that 5-MCDE induces AP-1 activation through the PI-3K-Akt pathway.

The role of another possible PI-3K downstream signaling pathway, mTOR-p70^{S6K}, in 5-MCDE-induced AP-1 acti-

vation was also investigated. Although the exact relationship between mTOR and PI-3K remains to be determined, mTOR has been found to be phosphorylated and activated by Akt in mitogen-stimulated cells and PTEN-deficient cells (Brunet et al., 1999; Scheid and Woodgett, 2001). Moreover, application of an mTOR inhibitor had antiproliferative and anticancer effects in PTEN-deficient cells (White, 1998; Laine et al., 2000). mTOR controls the mammalian translation machinery by activating p70^{S6K}, which enhances the translation of mRNA with 5' polypyrimidine tracts, as well as by inhibiting 4E-BP1 (also known as PHAS-1), which is a translation inhibitor binding to the CAP structure present at the 5' termini of mRNAs (Thomas and Hall, 1997; Hara et al., 1998). Currently, the mTOR derivative CCI-779 has entered a clinical trial for cancer treatment (Mills et al., 2001; Owa et al., 2001). However, we found mTOR is not involved in the 5-MCDE-induced AP-1 activation pathway in C141 cells. This notion was supported by the following evidence: (a) rapamycin can't decrease the 5-MCDE-induced AP-1 activation; (b) dominant-negative mutant p85 impaired 5-MCDE-induced AP-1 activation of all time points tested, and it only inhibited the early phase but not the late phase of p70^{S6K} phosphorylation; and (c) overexpression of dominant-negative mutant Akt impairs AP-1 activation, but not p70^{S6K} phosphorylation by 5-MCDE.

Besides the PI-3K pathway, MAPKs (ERKs, p38K, and JNKs) are also found to involve in AP-1 activation by many other stimuli. Serum and growth factors activate AP-1 transcriptional activity mainly through ERKs, whereas the induction of AP-1 by proinflammatory cytokines and genotoxic stress is mostly through activating JNKs and p38K cascades (Gruda et al., 1994; Hill et al., 1994; Chang and Karin, 2001). In this work, we demonstrated that PI-3K-Akt pathway mediated AP-1 activation specifically through activation of ERKs and JNKs. Although our most current data have shown that p38K are activated and involved in 5-MCDE-induced AP-1 activation (unpublished data), the signaling pathways leading to activation of p38K are still under investigation in our laboratory.

In summary, the present work demonstrates that 5-MCDE induces AP-1 activity in a dose- and time-dependent manner. AP-1 activation by 5-MCDE is mediated through a PI-3K/Akt/ERKs, JNKs-dependent, and mTOR-independent pathway. This conclusion is based on the results provided from using chemical inhibitors, including wortmannin, Ly294002, and rapamycin, or overexpression of dominant-negative mutants, such as $\Delta p85$, Akt-T308A/S473A, DN-ERK2, and DN-JNK1. This paper is highly relevant to the understanding of the molecular mechanisms involved in tumor promotion effects by 5-MCDE, but may also provide some useful information for cancer chemoprevention by enabling manipulation of the upstream regulatory signal pathways leading to AP-1 activation. Although mechanisms underlying the initiating signaling pathways by 5-MCDE are not well understood, we anticipate that oxidative stress, such reactive oxygen species, may be involved. This notion is supported by findings that 5-MCDE is capable of inducing oxidative DNA damage. Investigations are underway to determine if this is the case.

Materials and methods

Plasmids and reagents

CMV-neo vector plasmid, Akt mutant plasmid (SR α -Akt-T308A/S473A), dominant-negative mutant PI-3K (Δ p85), ERK2, and JNK1 plasmids were as described previously (Huang et al., 1996, 1997b, 1999a,b). The substrate for the luciferase assay was purchased from Promega; 5-MCDE was synthesized as described previously (Amin et al., 1985) and dissolved in DMSO to give a stock concentration at 2 mM. The phosphospecific antibodies against various phosphorylated sites of ERKs, p38K, and JNKs were obtained from New England Biolabs, Inc. FBS and MEM were purchased from BioWhittaker. The luciferase assay substrate was bought from Promega. Phosphospecific p70^{S6K} (Thr³⁸⁹) antibody, phosphospecific p70^{S6K} (Thr⁴²¹/Ser⁴²⁴) antibody, p70^{S6K} antibody phosphospecific Akt (Thr308) antibody, phosphospecific Akt (Ser473) antibody, and Akt antibody were obtained from New England Biolabs, Inc. The antibodies to Fra-1, Fra-2, Fos B, Jun B, and Jun D were obtained from Santa Cruz Biotechnology, Inc. The PI-3K inhibitors, wortmannin, and Ly294002 and the mTOR pathway inhibitor rapamycin were procured from Calbiochem. LipofectAMINE was obtained from GIBCO BRL.

Cell culture

The JB6 P⁺ mouse epidermal Cl41 cell line and its transfectants were cultured in monolayers at 37°C, 5% CO₂, using MEM that contained 5% FBS, 2 mM L-glutamine, and 25 μ g gentamicin per milliliter as described previously (Huang et al., 1997a, 1998; Watts et al., 1998). The cultures were dissociated with trypsin and transferred into new 75-cm² culture flasks (Fisher Scientific) from one to three times per week.

Generation of stable cotransfectants

Cl41 cells were cultured in a 6-well plate until they reached 85–90% confluence. 1 μ g of CMV-neo vector and 20 μ l of LipofectAMINE reagent, with 2 μ g of AP-1-luciferase reporter plasmid DNA, mixed with 12 μ g of dominant-negative mutant (SR α -Akt-T308A/S473A) or vector control were used to transfect each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM. Approximately 30–36 h after the beginning of the transfection, the cells were digested with 0.033% trypsin, and cell suspensions were plated into 75-ml culture flasks and cultured for 24–28 d with G418 selection (400 μ g/ml). The all stable clones (stable pool) in a flask were designed as a mass culture. The stable transfectants were identified by measuring both the basal level of luciferase activity and the blocking Akt activation. Stable transfectants, Cl41 AP-1 mass4 and Cl41 AP-1 Akt-T308A/S473A mass1, were established and cultured in G418-free MEM for at least two passages before each experiment.

AP-1 activity assay

Confluent monolayers of P⁺1-1 cells or Cl41 cell stable transfectants were trypsinized, and 8 \times 10³ viable cells suspended in 100 μ l MEM supplemented with 5% FBS were added to each well of 96-well plates. Plates were incubated at 37°C in a humidified atmosphere with 5% CO₂ in air. After the cell density reached 80–90%, cells were exposed to 5-MCDE at a final concentration as indicated in the figures for AP-1 induction. At different periods after treatment, the cells were extracted with lysis buffer (Promega) and their luciferase activity was determined by the Luciferase assay using a luminometer (Wallac 1420 Victor 2 multilable counter system; PerkinElmer) after the addition of 100 μ l of lysis buffer for 30 min at 4°C. The results are expressed as AP-1 activity relative to control medium containing the same concentration of DMSO only (relative AP-1 activity).

Northern blot

Whole RNA was extracted from monolayer cells with TRIzol[®] reagent according to the manufacturer's instructions (Invitrogen). 15 μ g RNA was run on a 1% agarose/formaldehyde gel at 120 V for 1.5 h. The RNA was transferred to a nylon membrane (Ambion) by vacuum for 1.5 h and cross-linked by UV wave. The murine *collagenase 1* (*MMP13*) probe was PCR-amplified from mouse mRNA (Yu et al., 2002). The probe was labeled with α -[³²P]dCTP using Prime-a-Gene Labeling System (Promega). The radioactivity was detected by phosphorimager using a scanner (model Storm[™] 860; Molecular Dynamics).

RT-PCR

The RNA was extracted by the Trizol[®] method mentioned in the previous paragraph. The cDNA was synthesized from 2 μ g RNA using First-Strand Synthesis System for RT-PCR according to manufacturer's instructions (Invitrogen). 1 μ l of synthesized cDNA was used for following multiplex PCR. The murine cyclin D1 (Klein et al., 2003) and β -actin (sense, 5'-CCT GTG

GCA TCC ATG AAA CT-3'; antisense, 5'-GTG CTA GGA GCC AGA GCA GT-3') primers were added together and PCR-amplified for 21 cycles. The PCR products were separated on 2% agarose gel.

PI-3K assay

PI-3K activities were assayed as described previously (Huang et al., 1996, 1997b). In brief, cells were cultured in monolayers in 100-mm dishes using normal culture medium. The media were replaced with 0.1% FBS MEM that contained 2 mM L-glutamine and 25 μ g of gentamicin per milliliter after the cell density reached 70–80%. 45 h later, the cells were incubated with fresh serum-free MEM medium for 3–4 h at 37°C. 5-MCDE was added to cell cultures for PI-3K induction. Cells were washed once with ice-cold PBS and lysed in 400 μ l of lysis buffer per plate (20 mM Tris, pH 8, 137 mM NaCl, 1 mM MgCl₂, 10% glycerol, 1% NP-40, 1 mM dithiothreitol, 0.4 mM sodium orthovanadate, and 1 mM PMSF). The lysates were centrifuged and the supernatants incubated at 4°C with 40 μ l of agarose beads (previously conjugated with the monoclonal anti-phosphotyrosine antibody Py20) overnight. Beads were washed twice with each of the following buffers: (1) PBS with 1% NP-40 and 1 mM dithiothreitol; (2) 0.1 M Tris, pH 7.6, 0.5 M LiCl, and 1 mM dithiothreitol; and (3) 10 mM Tris, pH 7.6, 0.1 M NaCl, and 1 mM dithiothreitol. Beads were incubated for 5 min on ice in 20 μ l of buffer 3, and 20 μ l of 0.5 mg/ml phosphatidylinositol (previously sonicated in 50 mM Hepes, pH 7.6, 1 mM EGTA, and 1 mM NaH₂PO₄) were added. After 5 min at RT, 10 μ l of the reaction buffer were added (50 mM MgCl₂, 100 mM Hepes, pH 7.6, and 250 μ M ATP containing 5 μ Ci of γ -[³²P]ATP), and beads were incubated for an additional 15 min. Reactions were stopped by adding 15 μ l 4 N HCl and 130 μ l of chloroform/methanol (1:1). After vortexing for 30 s, 30 μ l from the phospholipid-containing chloroform phase were spotted onto TLC plates coated with silica gel H containing 1.3% potassium oxalate and 2 mM EDTA applied in H₂O/methanol (3:2). Plates were heated at 110°C for at least 3 h before use. Plates were placed in tanks containing chloroform/methanol/NH₄OH/H₂O (600:470:20:113) for 40–50 min until the solvent reached the top of the plates. Plates were dried at RT and autoradiographed (Huang et al., 1996, 1997b).

Western blot

3 \times 10⁴ Cl41 transfectants were cultured in each well of 6-well plates to 70–80% confluence with normal culture medium. The cell culture medium was replaced with 0.1% FBS MEM supplemented with 2 mM L-glutamine and 25 μ g gentamicin per milliliter and cultured for 33 h. Cells were incubated in serum-free MEM for 3–4 h at 37°C, and were then exposed to 5-MCDE for various lengths of time. Cells were washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with one of the specific antibodies as indicated. The protein bands specifically bound to primary antibodies were detected using an anti-rabbit IgG-AP-linked and an ECF Western blotting system (Huang et al., 2002; Amersham Biosciences).

Statistical analysis

t test was used to determine the significance of differences in AP-1 activities between 5-MCDE treated and DMSO control. The differences were considered significant at a *P* < 0.05.

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