Alternariol induces DNA polymerase β expression through the PKA-CREB signaling pathway

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Abstract. Alternariol (AOH) is a mycotoxin of Alternaria alternata and can cause DNA damage and gene mutations. Low-dose and long-term treatment with AOH has been linked with incidence of esophageal carcinoma. DNA polymerase β (polβ) is a key enzyme in DNA base excision repair (BER). When it is overexpressed or mutated in cells, DNA polβ can cause genetic instability. Elevated DNA polβ has also been reported in several human cancers. Here, we report that AOH at 2, 10, 20 μM induces DNA polβ expression. In the process, protein kinase A (PKA) catalytic subunit activation, nuclear translocation and cAMP response element binding protein (CREB) phosphorylation are involved. AOH also increased CREB binding to the cAMP response element (CRE) consensus motif, which is present in the DNA polβ gene promoter. The PKA inhibitor H89 was able to block AOH-induced PKA-CREB activation, CREB DNA binding activity and decrease DNA polβ expression. Our results suggest that AOH can upregulate DNA polβ expression through the PKA-CREB signal transduction pathway.

Introduction

The genus Alternaria are wildly distributed in agricultural products such as wheat, tomatoes and apple juice (1,2). According to their different structures, mycotoxins of alternaria are classified into three types: tetramic acid derivatives, dibenzopyrone derivatives and perylene derivatives (2,3). Alternariol (AOH) is one of the important secondary products, belonging to dibenzopyrone derivatives, which is widely investigated on different aspects. AOH can cause frameshift mutations using Ames Salmonella strain TA97 (4). In cultured mammalian cells, AOH induced hypoxanthine-guanine phosphoribosyltransferase (HPRT) and thymidine kinase (TK) gene mutation (5). Our previous work also indicated AOH can cause DNA strands breaks (6). Squamous cell carcinoma of the fetal esophagus could be induced by AOH, and exposure to low level of AOH was associated with esophageal cancer (7,8).

Base excision repair (BER) is a process that recognize and repair DNA damage, which is caused by oxidant damage, and alkylation (9,10). BER is responsible for genetic stability (11,12). DNA polymerase beta (polβ) is a key enzyme responsible for BER (13). Polβ is a 39-kDa protein, which includes two domains, 8 and 31 kDa. The 8-kDa domain excises the 5-deoxyribose phosphate and the 31-kDa domain catalyses nucleotidyl to fill the gap (14,15). DNA polβ exists at relatively low level and constant expression in normal somatic cells (16). Endogenous and exogenous factor-caused DNA damage can induce DNA polβ expression. Overexpression of polβ was found in various human cancers such as esophageal carcinoma, gastric carcinoma, ovarian cancer (17-19). However, the pathway involved in polβ expression has not been fully investigated, and the role of polβ in tumorigenesis is still puzzling.

In the present study, the pathway involved in AOH induced polβ expression was investigated. We showed AOH-induced PKA activation and CREB phosphorylation, which related to polβ expression. Inhibition of PKA activity by H89 suppressed CREB phosphorylation and PKA-CREB-mediated polβ expression. These results indicated AOH could induce polβ expression via PKA-CREB signaling.

Materials and methods

Cell culture. NIH3T3 cells were cultured at 37˚C (5% CO_2) in RPMI-1640 with 10% fetal calf serum and 100 U/ml penicillin and 100 U/ml streptomycin.

Immunocytochemistry analysis. Cells were grown on glass coverslips in a 24-well plate and treated with drugs. The cells were fixed with 4% paraformaldehyde for 30 min. Cells were incubated with 3% H_2O_2 for 30 min, and were blocked with normal rabbit serum for 30 min to reduce non-specific binding, then incubated with anti-p-PKA catalytic subunit (1:200, Santa Cruz), anti-p-CREB (1:50, Cell Signaling) or anti-polβ antibody (1:200, Santa Cruz) overnight at 4˚C. After

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rinsing with PBS, the cells were incubated with biotin-labeled secondary antibody for 1 h at room temperature. Finally, the cells were stained with diaminobenzidine (DAB).

Immunofluorescence analysis. The steps were similar to immunocytochemistry procedures. FITC-conjugated goat anti-rabbit or donkey anti-goat IgG was used as secondary antibody and incubated with cells for 30 min without light. The cells were observed with a fluorescence microscope.

Western blotting. Cell lysate (50 μg) was separated on a 10% SDS-polyacrylamide gel and transferred to PVDF membrane. After blocking with 5% non-fat dried milk for 30 min, membrane was incubated with respectively primary antibody at 4˚C overnight (1:200 dilution for anti-PKA, 1:1,000 for anti-p-CREB, 1:200 for anti-polβ, 1:1,000 for anti-CREB). HRP-IgG secondary antibody was used for 2 h at room temperature. Protein bands were visualized by an enhanced chemiluminescence detection kit (ECL, Pierce). The bands were subjected to densitometry for quantitation using Bio-Rad Quantity One software.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared according to the protocol of nucleoprotein extraction kit (Beyotime). The sequence of the oligonucleotide of CRE was: 5'-TCGATTGGCTGACGTCAGAGAGAG-3'. As a control, the mutational CRE sequence: 5'-TCGATTGGC ACTCGTCAGAGAGAG-3' was employed. These oligonucleotides were labeled according to the protocol of the Biotin 3'-end DNA Labeling Kit (Beyotime). The single-stranded oligonucleotide probes were annealed to be double-strands. EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Beyotime). The specificity of binding was examined by competition with a 100-fold molar excess of unlabeled double stranded oligonucleotide added to the mixture. For supershift assays, the nuclear extract was incubated for 20 min at room temperature with labeled probe and antibodies against phosphorylated CREB (Ser133) (Cell Signaling). The complexes of oligonucleotide probes and nuclear protein were separated from the unbound probes by electrophoresis with 6% polyacrylamide gels. A transfer of the complex and free probes from the polyacrylamide gel onto the nylon membrane was performed by the semi-dry transfer method. The membranes were visualized with ECL.

Statistical analyses. All results are presented as mean ± SEM, statistical analysis was performed by ANOVA in SPSS 11.0 software. $P<0.05$ was considered to indicate statistical significance.

Results

AOH-induced DNA pol β expression. To investigate whether AOH can induce polβ expression or not, NIH3T3 cells were treated with AOH. Immunocytochemistry assay and Western blotting was performed to determine polβ protein level after different dose of AOH treatment. In immunocytochemistry assay, brown coloration was obviously seen in cell nucleus of 10 and 20 μM AOH-treated groups (Fig. 1A). Western blotting results exhibited that the protein level of polβ increased in a dose-dependent manner with AOH treatment (Fig. 1B).

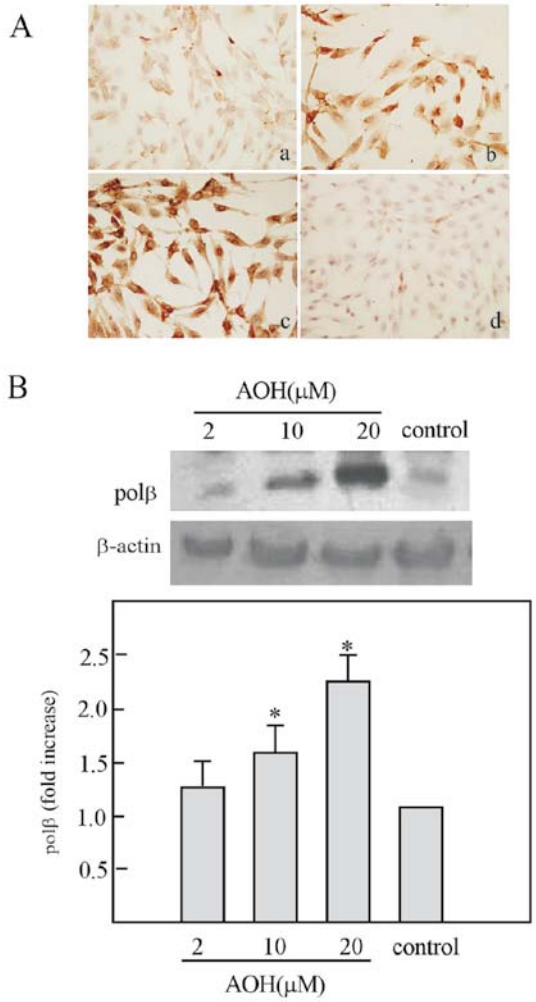

Figure 1. AOH induces DNA polβ expression. NIH3T3 cells were treated with 2, 10, 20 μM AOH and DMSO respectively for 16 h. (A) Immunocytochemistry analysis showed polβ expression in 2, 10, 20 μM AOH and control group: (a) 2 μM AOH; (b) 10 μM AOH; (c) 20 μM AOH; (d) control. (B) Western blotting results showed polβ expression increased after AOH treatment. Data are shown as means ± SD of values from triplicate experiments. *Significant ($P<0.05$) change in expression compared to DMSO treatment.

AOH-induced activation of PKA and nuclear translocation. PKA activation was induced by 2, 10, 20 μM AOH for 1 h in immunocytochemistry experiments, brown coloration was obviously seen in cell nucleus of the 10 and 20 μM AOH-treated groups (Fig. 2A). Immunofluorescence analysis also indicated the nuclear translocation of activated PKA increased after 20 μM AOH treatment compared with control group (Fig. 2E).

Western blotting was used to determine the level of PKA activation after AOH treatment at different time-point. Results showed the activation of PKA increased in a dose-dependent manner (Fig. 2B). The level of PKA activation was the highest when the NIH3T3 cells were treated with 20 μM AOH at 60 min (Fig. 2C). H89, an inhibitor of PKA, can effectively block PKA activation (20). To confirm whether AOH induced PKA activation, we pretreated the NIH3T3 cells with 10 μM H89 for 1 h, and then treated the cells with 20 μM AOH. Immunofluorescence analysis showed a significant decrease of PKA actvation in H89 pretreatment group compared with the AOH-treated group (Fig. 2E). Similar results were observed in

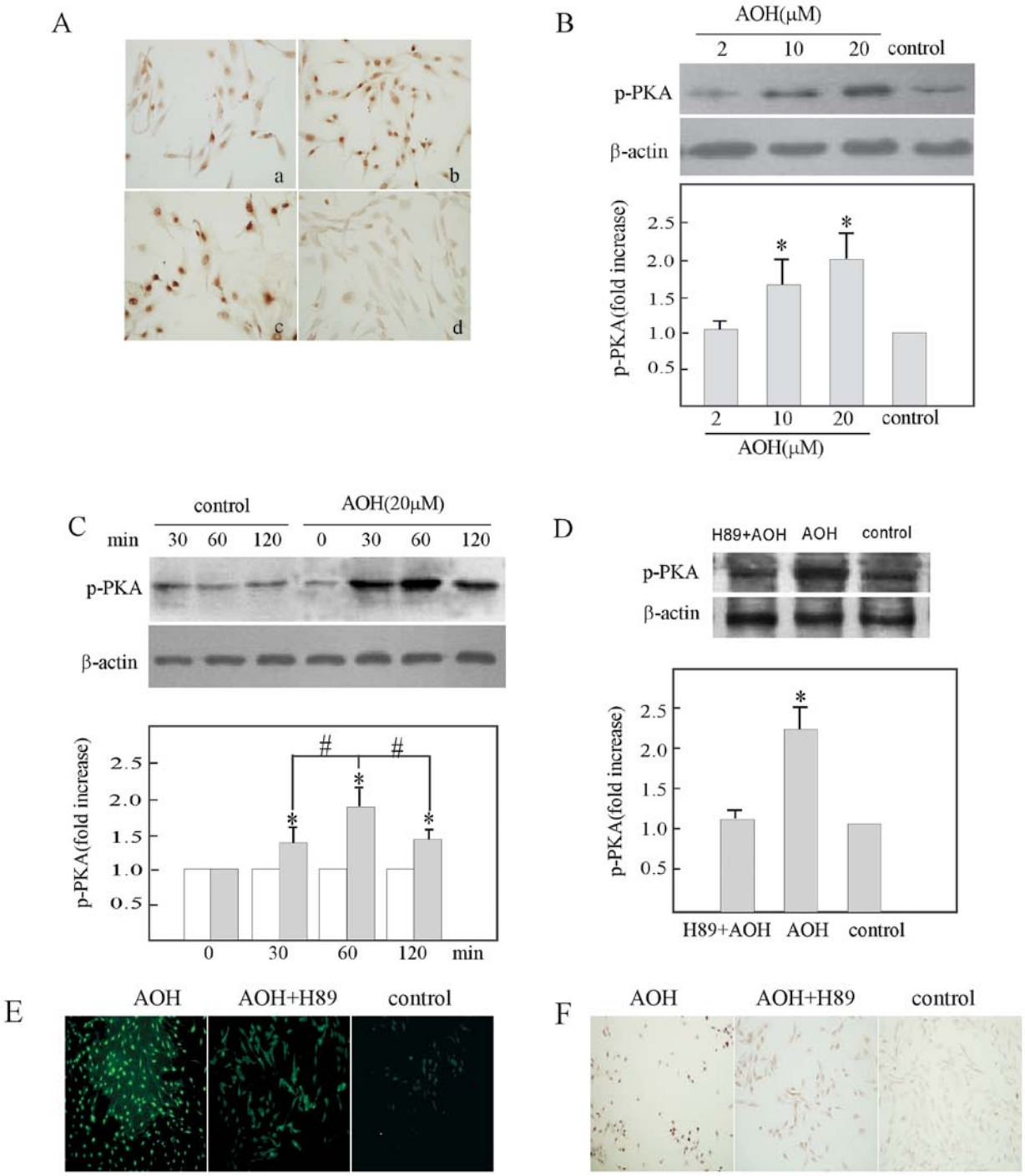

Figure 2. AOH-induced PKA catalytic subunit activation and translocation. NIH3T3 cells were treated with indicated dose of AOH for 1 h. The levels of phosphorylated PKA catalytic subunit were visualized by immunocytochemistry analysis (magnification x400) (A) and Western blotting (B). The cells were treated with AOH for the indicated time, and the levels of phosphorylated PKA catalytic subunit were measured by Western blotting (C). Cells were pretreated with H89 (10 μM) 1 h prior to 20 μM AOH exposure. The results indicated by Western blotting (D) immunofluorescence analysis (magnification x200) (E) and immunocytochemistry analysis (magnification x200) (F). Data are shown as means ± SD of values from triplicate experiments. *Significant ($P<0.05$) change in expression compared to DMSO treated control. #Significant ($P<0.05$) change in expression compared to the group of AOH treatment for 60 min.

Western blotting (Fig. 2D) and immunocytochemistry analysis (Fig. 2F).

AOH-induced activation of CREB. PKA is an important regulator of CREB activity. PKA can phosphorylate CREB at Ser133 and activate it fully (21). To determine whether AOH-mediated PKA activation can phosphorylate CREB in NIH3T3 cells, we investigated phosphorylation status of CREB on Ser133. Immunocytochemistry analysis showed CREB phosphorylation increased in 10 and 20 μM AOH-treated groups (Fig. 3A). Western blot analysis also demonstrated that AOH induced CREB phosphorylation in a dose-dependent manner (Fig. 3B and C). The phosphorylation peaked at 2 h after 20 μM AOH treatment (Fig. 3C). The phosphorylation level decreased after

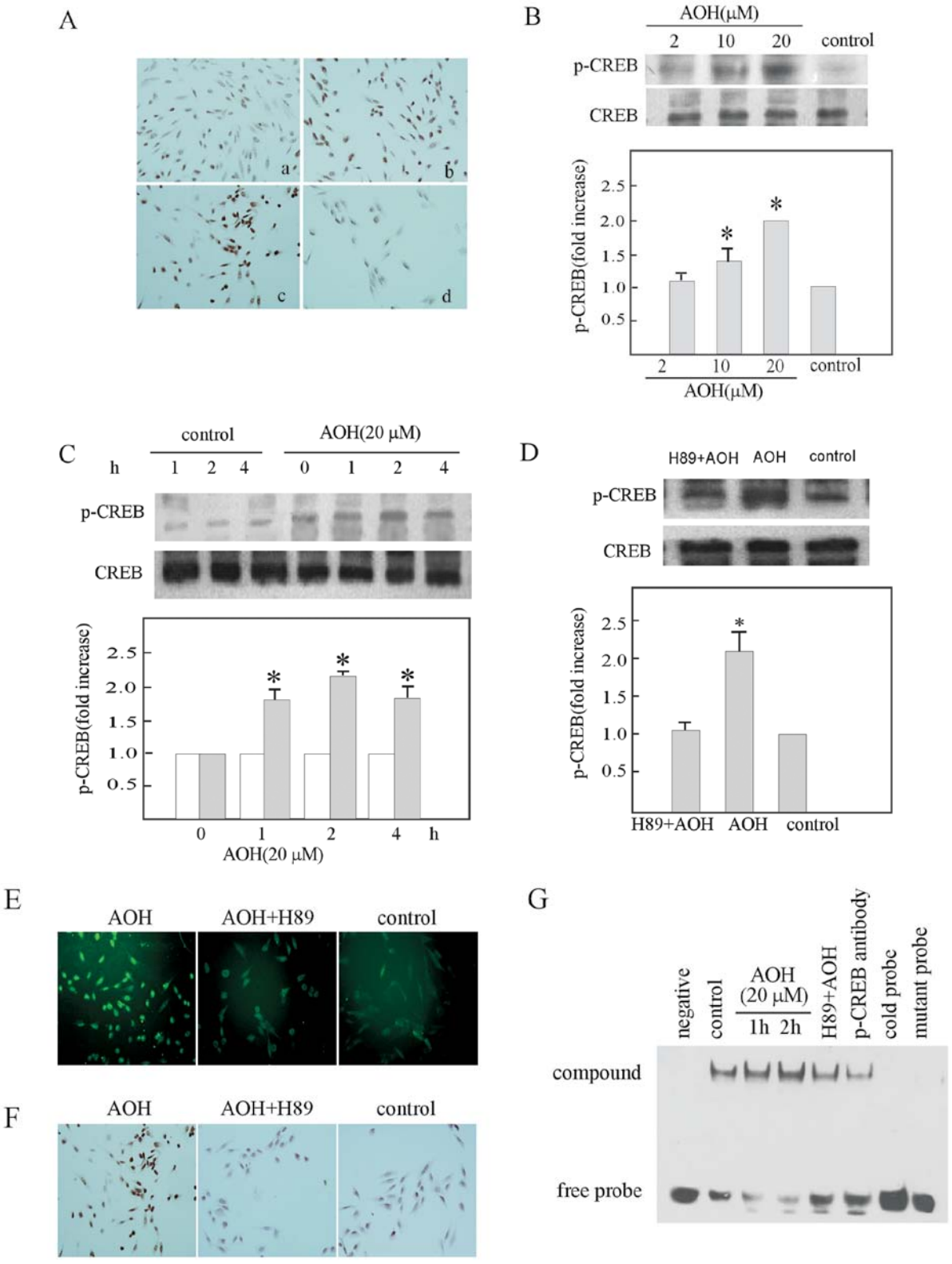

Figure 3. AOH-induced phosphorylation of CREB. NIH3T3 cells were treated with indicated dose of AOH for 2 h. The levels of phosphorylated CREB were visualized by immunocytochemistry analysis (magnification x100) (A) and Western blotting (B). The cells were treated with AOH for 1, 2 and 4 h and the levels of phosphorylated CREB were measured by Western blotting (C). Cells were pretreated with H89 (10 μM) for 1 h prior to 20 μM AOH exposure. The results were measured by Western blotting (D), immunofluorescence analysis (magnification x200) (E) and immunocytochemistry analysis (magnification x200) (F). The p-CREB DNA binding activity was detected by EMSA (G). Data are shown as means ± SD of values from triplicate experiments. *Significant ($P<0.05$) change in expression compared to DMSO treated control.

H89 (10 μM) treatment (Fig. 3D, E and F). These results indicated that AOH stimulated PKA activation followed by CREB phosphorylation.

Phosphorylation of CREB at Ser133 closely related with its DNA binding activity. Next, we checked whether AOH treatment can increase CREB DNA binding activity in NIH3T3

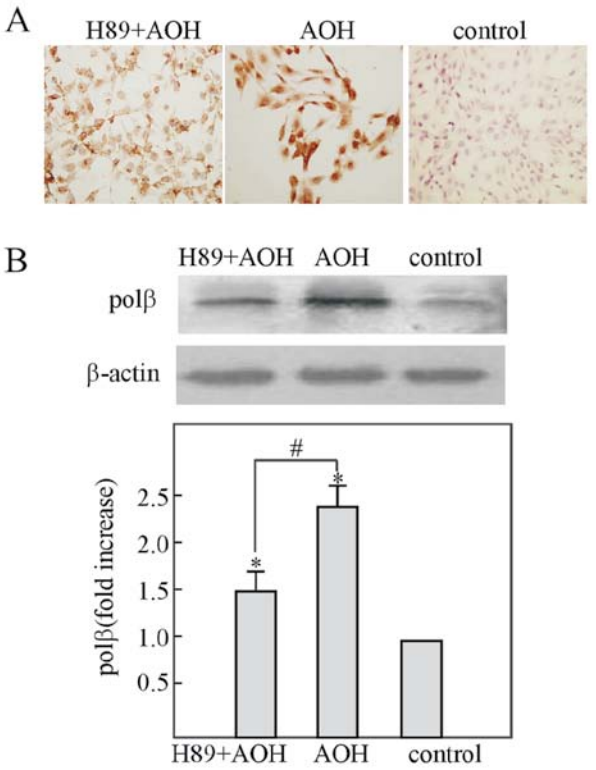

Figure 4. H89 inhibited the expression of DNA polβ. NIH3T3 cells were treated with H89 prior to AOH treatment. DNA polβ expression was detected by immunocytochemistry analysis (magnification x400) (A) and Western blotting (B). Data are shown as means ± SD of values from triplicate experiments. *Significant (P<0.05) change in expression compared to DMSO treated control. #Significant (P<0.05) change in expression compared to H89 pretreatment group.

cells. Cells were treated with AOH (20 μM) for various time periods and then the activity of CREB was detected by EMSA. The result indicated AOH treatment at 1 and 2 h, respectively, increased CREB DNA binding activity compared to DMSO-treated control. CREB DNA binding activity was partly inhibited by pretreatment with H89 (Fig. 3G). These results demonstrated phosphorylated CREB stimulated by AOH strongly binded to cAMP response elements containing the 5'-TGACGTCA-3' consensus motif. The specificity of binding was examined by competition with the unlabeled probes (cold probes) and supershift assays.

AOH up-regulated polβ expression via the activated PKA-CREB pathway in NIH3T3 cells. AOH can cause DNA damage and stimulate DNA polβ expression. To check whether this procedure is through PKA-CREB signaling transduction pathway NIH3T3 cells were treated with DMSO, AOH (20 μM) and AOH together with H89 (20 μM AOH and 10 μM H89) for 16 h. Western blot and immunocytochemistry analysis both indicated that AOH led to a significant increase in polβ protein level and H89 treatment inhibited AOH-induced polβ protein level (Fig. 4). The results were consistent with PKA and CREB activation.

Discussion

In mammalian cells, DNA damage is an important event which arouses cell stress reaction. DNA damage repair systems can be initiated by DNA damage. Base excision repair (BER) is a process that recognizes and repairs damaged or modified bases, including oxidative base damage, multiple forms of alkylation damage and apurinic/apyrimidinic (AP) sites formed by the spontaneous loss of bases (22,23). DNA polβ is a nuclear enzyme involved in the base excision and single-stranded DNA break repair pathways and long patch base excision repair (24,25). Studies have indicated DNA polβ mutation in esophageal, colorectal and prostrate cancer (26-28). DNA polβ overexpression was also found in various tumors indicating that DNA polβ is closely related with tumorigenesis.

AOH was found closely related to esophageal tumorigenesis in our recent study after exposure of NIH3T3 cells to 10 μM AOH (6). The data demonstrated that AOH caused tumorigenesis probably related to DNA damage and repair system. In this study, we found that polβ expression was increased after AOH treatment (Fig. 1). These results might indicate that overexpression of polβ participated in the AOH-induced DNA damage repair.

Various signal transduction pathways are involed in tumorigenesis, including MAPK, PI3K/Akt, Notch, PKA-CREB pathway. In PKA lacking cells MNNG failed to activate the polβ promoter (29). PKA comprises of two catalytic (C) subunits and two regulatory (R) subunits (30). The activity of the C subunit is regulated by a set of four different regulatory R subunit isoforms. When C subunit is activated, it separates from R subunits and translocates into nucleus (30). To explore the role of PKA activation in AOH-induced polβ expression, we detected PKA C subunit activation after AOH treatment. The results showed AOH initiated PKA C subunit activation and made its C subunit to translocate to the nucleous (Fig. 2A and E). Moreover, PKA inhibitor H89 could inhibit AOH-mediated PKA activation and the nuclear translocation (Fig. 2E and F).

PKA have various downstream targets involved in different pathways. CREB is an important target of PKA. CREB is an important transcription factor which include a basic region leucine zipper (bZIP). CREB become an activator in phosphorylation state by PKA. Phosphorylated CREB binds via its bZIP domain as a dimer to cAMP response elements (CREs) containing the 5'-TGACGTCA-3' consensus motif (31,32). To identify the function of PKA-CREB signaling pathway in polβ expression, we checked the effect of AOH on CREB phosphorylation. AOH can induce CREB phosphorylation at Ser133, the peak of CREB phosphorylation emerged later than the peak of PKA after AOH treatment. In addition, PKA inhibitor H89 blocked CREB phosphorylation and inhibited expression of polβ. To investigate CREB-CRE binding activity when exposured to AOH, we conducted EMSA. The result indicated that AOH strongly rendered the binding activity of CREB-CRE. These data showed that p-CREB was involved in AOH-induced DNA polβ expression.

The sequence of DNA polβ core promoter has three GC boxes and the decanucleotide palindromic sequence GTGACGTCAC at positions -49 to -40 (33). The element is similar to the ATF/CREB transcription factor-binding site. The transcriptional activation of polβ promoter is exhibited strongly by DNA damaging agent MNNG treatment of CHO cells (33,34). Mutated polβ promotor fusion genes lacking the element failed to bind protein at this site and failed to respond to MNNG treatment of cells (29). It was shown that the Ser133

phosphorylated CRE binding protein (CREB) was higher versus control in vero cells after 60 min of MNNG treatment (35,36). The results suggested MNNG-induced polβ expression might be mediated by PKA-CREB signaling pathway. In the present study, we examined the effects of AOH on PKA activation, CREB phosphorylation and polβ expression. AOH can stimulate PKA activation, CREB phosphorylation and increase polβ expression. PKA inhibitors can decrease CREB activation and inhibit DNA polβ expression.

In conclusion, these data suggest AOH induced DNA polβ expression through PKA-CREB signaling pathway. This procedure probably involved AOH induced tumorigenesis.

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