Direct regulation of caspase-3 by the transcription factor AP-2α is involved in aspirin-induced apoptosis in MDA-MB-453 breast cancer cells

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Abstract. Aspirin has been reported to trigger apoptosis in various cancer cell lines. However, the detailed mechanisms involved remain elusive. The present study aimed to investigate whether aspirin plays a role in apoptosis of MDA-MB-453 cells. The effect of aspirin on the proliferation of human MDA-MB-453 cells breast cancer cells was evaluated using MTT assay, flow cytometry and western blotting. The present study reports that aspirin induces the apoptosis of MDA-MB-453 breast cancer cells which was attributed to the increased expression and activation of caspase-3. Moreover, AP-2α, a transcription factor highly expressed in MDA-MB-453 cells, was identified as a negative regulator of caspase-3 transcription and AP-2α was attenuated following aspirin treatment. Therefore, aspirin may increase the expression of caspase-3 by inducing the degradation of AP-2α, which increases activated caspase-3 expression, thereby triggering apoptosis in MDA-MB-453 cells. Thus, aspirin may be used in breast cancer therapy.

Introduction

Aspirin (acetylsalicylic acid) has been used as a treatment for high body temperatures, headaches and muscle pain for more than a century. Accumulating evidence from large-scale epidemiological studies, animal models and in vitro experiments suggest that regular use of aspirin may reduce the risk of cancer by inducing cancer cell apoptosis (1-3). Findings of recent studies have indicated that aspirin is able to block NF-κB activation (4,5), induce iNOS to produce NO (6,7), inhibit COX-2 (8), ErbB2 (9) and Bcl-2 (10) and acetylate or phosphorylate p53 (3,11), inducing cell apoptosis or proliferation inhibition. However, the cell pathways through which aspirin exerts its anticancer effects are not well understood and require further elucidation.

Apoptosis in anticancer strategies is a multi-step process involving the activation of caspases, a family of cysteine proteases. There are two types of apoptotic caspases: initiators (caspases-2,-8,-9 and -10) and effectors (caspases-3,-6 and -7). Initiator caspases cleave inactive pro-forms of effector caspases to activate them and then effector caspases catalyze the specific cleavage of other key cellular proteins to trigger the apoptotic process. In aspirin-induced cancer cell apoptosis, caspase-3 and other caspase family members were previously demonstrated to be crucial upregulated factors (4,10,12,13).

The transcription factor AP-2 family, which consists of AP-2α, β, γ, δ and ε, regulates the transcription of numerous genes involved in mammalian development, the cell cycle, cell proliferation, apoptosis and carcinogenesis by binding to the genes’ promoter regions (14,15). Several reports have suggested that AP-2α and AP-2γ are marked functional activators for ErbB2 overexpression in mammary carcinoma (16,17), while ErbB2 overexpression is associated with increased tumorigenicity, enhanced metastasis, poor prognosis and decreased chemosensitivity (18).

The aim of the present study was to demonstrate whether aspirin induces apoptosis in MDA-MB-453 cells by upregulating caspase-3 expression and activity. Caspase-3 was found to be directly and negatively regulated by AP-2α, which is degraded following aspirin treatment. The caspase-3 pathway appeared to participate in aspirin-induced apoptosis of MDA-MB-453 cells through the downregulation of AP-2α.

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Materials and methods

Plasmids, siRNA and reagents. The full-length coding region of AP-2α was cloned into a pCMV-Myc plasmid (Clontech, Mountain View, CA, USA). The promoter region (nt -799 to nt +93 from the transcription start codon) of caspase-3 was generated by PCR (19) and cloned into the luciferase reporter plasmid pTAL-luc (Clontech), denoted pTAL-luc-caspase-3. The primer sequences used were: 5'-CGGGTACCC TTATTTCTCATGATGTT-3' (forward) and 5'-GAAATCG TGCCCTCTCATCACCACT-3' (reverse). Single or double AP-2α binding site mutants were created by site-directed mutation and denoted as pTAL-luc-M1, -M2 and -M3. Two specific small interfering RNAs (siRNAs) against AP-2α were purchased from GenePharma Co. (Shanghai, China). The two siRNA sense sequences are 5'-UUUCUCAACCGACAAAAU-3' and 5'-CGAGUCUCGUUCAGUCAGU-3'. Aspirin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and solubilized in water.

Cell culture, transfection and treatment. MDA-MB-453 cells were cultured in DMEM (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37˚C in a 5% CO₂ incubator. Cells were cultured to 80% confluence and transiently transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Aspirin treatment proceeded at 90% confluence continuously in low-serum (0.5% FBS) medium for 24 h.

Cell proliferation assay. Inhibition of cell proliferation was evaluated by the MTT assay (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, MDA-MB-453 cells were placed in 24 well plates at a density of 1x10⁵ cells/well in 450-µl medium and 24 h after attachment, the cells were treated with 0-20 mM aspirin for a further 24 h. Then, 50 µl of MTT (5 mg/ml in PBS) solution were added to each well and the cells were incubated for a further 4 h, followed by the addition of 150 µl of dimethyl sulfoxide (DMSO; Sigma-Aldrich)/water. The cells were left for 30 min at room temperature to allow color development. Absorbance values were determined using an enzyme-linked immunosorbent assay (ELISA) reader (Model 680; Bio-Rad, Hercules, CA, USA) at 570 nm.

Flow cytometric analysis of apoptosis. Apoptotic and total dead cells were stained by the Annexin V-FITC/propidium iodide (PI) detection kit (Bender MedSystems, Vienna, Austria) according to the manufacturer’s instructions. In brief, MDA-MB-453 cells (1x10⁵ cells/well) were washed with 1X Annexin V binding buffer and stained with Annexin V (5 µl) and PI (10 µl) for 15 min at room temperature in the dark. Following the addition of 400 µl of binding buffer, cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Caspase-3 activity assay. The activity of caspase-3 was determined using the Caspase-3 Activity Detection kit (Beyotime Institute of Biotechnology, Haimen, China). To evaluate the activity of caspase-3, cell lysates were prepared following the designated treatment. Assays were performed in 96-well plates by incubating 10 µl protein of cell lysate/sample with 10 µl caspase-3 substrate (Ac-DEVD-pNA, 2 mM) in 80 µl of reaction buffer [1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM NaCl and 10% glycerol] at 37˚C for 5 h. Absorbance values were determined using an enzyme-linked immunosorbent assay (ELISA) reader (Model 680; Bio-Rad) at 405 nm.

The detailed procedure including the standard curve preparation was described in the manufacturer's instructions. All the experiments were performed in triplicate.

RNA extraction, semi-quantitative RT-PCR and real-time PCR. Total RNA extraction, RT- and real-time PCR were performed as described previously (9). The real-time PCR primers used were: AP2α 5'-CTCAACCGACAAATCTC3' (forward) and 5'-CGGTGAACTCTTTGCATATC-3' (reverse); caspase-3 5'-CAGTGGAGGCCGACTTC3' (reverse) and 5'-TGCGCAAAGCGACTGGATT3' (reverse). For semi-quantitative RT-PCR, DNA was amplified under the following conditions: denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec and the number of cycles for amplification was 21-25. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Western blotting. Cells were lysed in RIPA buffer with protease inhibitors (Sigma-Aldrich). Equal amounts of protein were separated on a 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After blocking, the PVDF membranes were washed three times for 10 min with TBST at room temperature and incubated for 1-2 h at room temperature with TBST-diluted primary antibodies, anti-AP2α (1:500) and anti-caspase-3 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following extensive washing, the membranes were further incubated with the secondary peroxidase-labeled antibodies (1:2,000) in 5% non-fat dry milk/TBST for 1 h. The membranes were again washed three times for 10 min with TBST at room temperature, immunoreactivity was visualized using the enhanced chemiluminescence ECL kit (Pierce Biotechnology, Rockford, IL, USA) and the membranes were exposed to Kodak film. The membranes were then stripped and reprobed with anti-GAPDH antibody (1:1,000; Santa Cruz Biotechnology, Inc.) as a loading control.

Chromatin immunoprecipitation (ChiP) assay. The ChiP assay was performed using the EZ ChiP™ kit (Millipore) according to the manufacturer's instructions, as previously described (22). The antibodies used included anti-AP2α antibody (Santa Cruz Biotechnology, Inc.) and normal rabbit IgG (Millipore). The caspase-3 promoter ChiP primers used in the present study were: 5'-AACACAGCATGCGTGGACCT-3' (forward) and 5'-GCCTCCTCATACCATCACCACT-3' (reverse).

 Luciferase reporter assays. MDA-MB-453 cells were seeded at 5x10⁴ cells/well in 12-well plates and transfected at 80% confluence using Lipofectamine 2000 reagent according to the manufacturer's instructions. After 24 h of transfection, the luciferase activity was measured using the luciferase reporter assay system (Promega, Madison, WI, USA).
Statistical analysis. Results of bar graphs were expressed as the mean ± SD obtained from three independent experiments. Statistical differences were evaluated using the Student’s t-test. *P<0.05 was considered to indicate statistically significant differences.

Results

Aspirin induced apoptosis and upregulated the expression and activity of caspase-3 in MDA-MB-453 cells. To evaluate the effect of aspirin on the proliferation of human MDA-MB-453 breast cancer cells, the MTT assay was used and the results indicated that aspirin decreased cell proliferation in a dose- and time-dependent manner (Fig. 1A). A significant antiproliferative effect of aspirin appeared following treatment with 20 mM for various time periods. The percentage of viable cells decreased to 62.38% under treatment with 20 mM of aspirin for 24 h and then to 18.97% after 72 h treatment, compared with the controls. To investigate whether aspirin induced cell apoptosis and death, cells were treated with various concentrations (0-20 mM) of aspirin for 24 h and then subjected to Annexin V/PI-based flow cytometry. Exposure to aspirin significantly affected apoptosis in the MDA-MB-453 cells (Fig. 1B). When exposed to 20 mM aspirin for 24 h, the percentage of apoptotic cells reached 12.98%, notably higher than the untreated cells. As other studies have described upregulation of the expression and activity of caspase-3 in gastric, cervical and prostate cancer cell apoptosis (12,13,23,24), the expression of caspase-3 was investigated by real-time PCR and western blotting. As shown in Fig. 1C and D, the mRNA and protein levels of caspase-3 were increased in a dose-dependent manner when exposed to 0-20 mM aspirin for 24 h. Moreover, to investigate the expression of activated caspase-3, the activity of caspase-3 was evaluated and the results showed that activity was increased in a similar dose-dependent manner in response to aspirin treatment for 24 h (Fig. 1E). These data suggested that aspirin induced apoptosis in MDA-MB-453 cells via caspase-3 upregulation.

AP2α directly binds to the promoter region of caspase-3 in MDA-MB-453 cells. Since the transcription level of caspase-3 is upregulated in aspirin-treated MDA-MB-453 cells, the promoter region of caspase-3 was analyzed by the JASPAR and MatInspector programs. As shown in Fig. 2A, two AP-2α consensus DNA-binding sites in the promoter region of caspase-3 (nt -901 to nt +200 from transcription start codon) were observed, nt -227 and nt -107. As AP-2α is important in mammary carcinoma and highly expressed in MDA-MB-453 cells (16,17,25), the direct binding of AP-2α to the caspase-3 promoter was demonstrated by ChIP experiments.
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The predicted band was detected in the input and AP-2α-ChIP-derived DNA samples, but not in the control IgG-ChIP-derived DNA samples. To confirm the precise binding sites of AP-2α on the caspase-3 promoter, the wild-type binding sites of AP-2α are indicated by ovals, and the mutated sites are indicated by crosses (×). The data in the bar charts are the mean ± SD of three independent experiments.

AP2α negatively regulated caspase-3 in MDA-MB-453 cells. To determine whether this binding conferred positive or negative regulation on caspase-3, the expression of AP-2α was knocked down in MDA-MB-453 cells by siRNA and the expression of caspase-3 was detected using semi-quantitative RT-PCR and western blotting. Cells transfected with siRNAs against AP-2α showed clear increases in caspase-3 mRNA (Fig. 3A) and protein levels (Fig. 3B). Expression of the activated caspase-3 was also enhanced, as determined by a caspase-3 activity assay (Fig. 3C). However, overexpression of AP-2α decreased the expression levels (Fig. 3A and B) and activity (Fig. 3C) of caspase-3, whereas the controls did not change.

Aspirin induced the degradation of AP-2α through the proteasome pathway in MDA-MB-453 cells. To investigate whether AP-2α responds to aspirin treatment in MDA-MB-453 cells,
the AP-2α expression levels of mRNA and protein were evaluated. The results showed that the protein level of AP-2α was downregulated in a dose-dependent manner in MDA-MB-453 cells following exposure to various concentrations of aspirin for 24 h (Fig. 4A). However, no significant changes were observed in the mRNA levels in the semi-quantitative and real-time PCR experiments (Fig. 4B and C). It appeared that aspirin did not affect AP-2α expression at the transcriptional level.

A previous study reported that aspirin treatment affects protein proteasomal degradation in breast cancer cells (26). MG132, a proteasome inhibitor, was used to evaluate whether it was able to block the degradation of AP-2α in MDA-MB-453 cells following aspirin treatment. Cells were pre-incubated with MG132 (20 μM) for 5 h prior to aspirin (10 mM) treatment for 24 h and cell extracts were used for western blotting to detect AP-2α expression. As shown in Fig. 4D, the degradation of AP-2α protein was blocked in the presence of MG132, suggesting that aspirin induced AP-2α degradation via a proteasome-mediated pathway.

**Discussion**

Aspirin has multiple effects and is involved in various cellular processes. In the last few years, several studies have shown that aspirin inhibits cancer cell proliferation and induces apoptosis (3,11,24), which is consistent with the present results that aspirin exhibits the same activity in MDA-MB-453 breast cancer cells in a dose- and time-dependent manner. Although the inhibition of certain proteins, such as COX and NF-κB, may contribute to the anticancer effects of aspirin (8,27), at present the pathways leading to these effects remain to be determined.

In the present study, several novel observations were reported, including a mechanism by which aspirin may exert its anticancer effects. The present results showed that the expression and activity of caspase-3 were upregulated following aspirin treatment, suggesting that the induction of apoptosis by aspirin in MDA-MB-453 cells may depend on the caspase pathway and caspase-3 was regulated at its transcriptional level. Promoter analysis then revealed that AP-2α may be a regulator of caspase-3. Considering the important roles of AP-2α in breast cancer and its high expression levels in several breast cancer cell types including MDA-MB-453 (16,25), further experiments were performed to study the association between AP-2α and caspase-3. The data showed that AP-2α negatively regulates caspase-3 transcription via binding to the promoter region of caspase-3, thus downregulating the expression of caspase-3 and decreasing its activity. The expression of AP-2α in cells treated with aspirin was then evaluated. The present results have shown that AP-2α was downregulated following aspirin treatment at the protein but not mRNA level. Aspirin has been reported to affect protein degradation through the proteasome pathway (26). To assess whether this downregulation of AP-2α depends on the proteasome pathway, MG132 was selected to block the proteasome pathway. MG132 abrogated the aspirin-induced reduction of AP-2α.

From the present study, a working model may be proposed. In this model, AP-2α negatively regulates caspase-3 by binding to its proximal promoter region in AP-2α-positive cancer cells. Aspirin promotes the proteasome pathway-dependent degradation of AP-2α, which increases the expression of apoptotic effector caspase-3, leading to an increase in activated caspase-3 and finally causing cell apoptosis. This suggests a novel mechanism for aspirin in breast cancer treatment.

AP-2α acts as an oncogene, which is consistent with previous reports (16,17,25,28). However, there are also several lines of evidence indicating that AP-2α may act as a tumor suppressor gene. Overexpression of AP-2α induces apoptosis in a number of cancer cells (29-31). AP-2α has been shown to act as both a tumor suppressor and an oncogene in various cancer types that may depend on the expression level and signaling pathways AP-2α is involved in. The controversy concerning the role of AP-2α reflects the complexity of the cell signaling pathways. However, a number of critical questions remain to be answered in order to understand the mechanisms of aspirin-induced apoptosis.
In summary, the present study demonstrated for the first time that aspirin upregulates caspase-3 activity through the downregulation of AP-2α gene expression, leading to the apoptosis of human breast cancer cells. The present findings also suggest that aspirin is a potentially powerful therapeutic agent for various AP-2α-dependent human cancers.

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