Abstract. Adrenocorticotrophic hormone (ACTH)-producing pituitary adenoma leads to excess ACTH secretion, which is associated with significant mortality and impaired quality of life. Thus far, the first line therapy is the transphenoidal microsurgery. Considering the high recurrence rate and complications of surgery, novel agents, which directly target on pituitary ACTH-producing adenoma and suppress ACTH secretion are urgently required. In the present study, the effect of ursolic acid (UA) as a candidate agent targeting ACTH-producing AtT20 cells was investigated. It was demonstrated that UA inhibited the viability and induced apoptosis of AtT20 cells and decreased ACTH secretion. The process of apoptosis involved a decrease of the B cell lymphoma 2 (Bcl-2)/Bcl2-associated X protein ratio followed by a release of mitochondrial cytochrome c into the cytosol with subsequent activation of caspase-9, -3/7 and -8. The potential signaling pathway involved the activation of c-Jun N-terminal kinase (JNK) but not extracellular signal-regulated protein kinases1/2 and p38 mitogen-activated protein kinase. The JNK pathway participated in UA-induced mitochondrial apoptotic signaling transduction via increasing the phosphorylation and degradation of Bcl-2, which may be partly attenuated by the JNK inhibitor SP600125. In conclusion, the present study indicates that UA may be a promising candidate agent for the management of ACTH-producing pituitary adenoma.

Introduction

Adrenocorticotrophic hormone (ACTH)-producing adenoma, accounting for 10-15% of pituitary tumors, result in Cushing’s disease due to excessive secretion of ACTH and, consecutively, cortisol (1). Cushing’s disease possesses a high morbidity and mortality rate if managed inadequately (1-3). Pituitary surgery, particularly transphenoidal microsurgery, remains the first choice amongst therapies. However, complication of hypopituitarism occurs in ~80% of all cases (4) and the risk of recurrence reaches 20-25% at 10 years following surgery (5). Radiation therapy, which is often used in cases of persistence or recurrence, is limited by the risk of necrosis in the temporal lobe of the brain and high occurrence of long-term hypopituitarism (6). Medical therapy with classic adrenal-directed drugs (steroidogenesis inhibitors) may be highly effective but with severe side-effects, and these agents cannot inhibit underlying tumors or restore normal secretory dynamics (7-9). Pituitary-directed drugs, including somatostatin analogs and dopamine agonists, demonstrate certain effects on ACTH secretion in Cushing’s disease; however, further long-term trials are required to determine the safety and efficacy (10-12). Other agents, such as peroxisome proliferator-activated receptor γ (PPAR-γ) agonists for the treatment of Cushing’s disease, are experimental and not currently available in human clinical trials (13,14). As noted, the drugs that directly target the pituitary tumor growth and ACTH secretion are urgently required and would be an attractive option in the medical management of ACTH-producing pituitary adenoma.

Ursolic acid (UA) is a triterpenoid compound widely distributed in food, medicinal herbs and other plants (15). UA has various pharmacological properties, including anti-oxidant, anti-inflammatory and anti-hyperlipidemic activities (16,17). In addition, UA has clinical applications for treating tumor patients as a promising antitumor agent (18). It has been proven that UA inhibited tumorigenesis and progression in a broad spectrum of tumors, including hepatocellular carcinoma, melanoma, and prostate, colorectal, breast and bladder cancer (19-26). The reported molecular
mechanisms involved in UA-induced apoptosis include inhibition of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) activity and protein tyrosine kinase (27,28). However, there is no report in the literature on the effect of UA on ACTH-producing pituitary adenoma and its potential molecular mechanisms.

In the present study, the effect of UA on apoptosis and ACTH secretion in AtT20 cells was investigated. The potential underlying molecular mechanisms of action, including endogenous, exogenous signaling pathways and the JNK pathway, were also explored.

Materials and methods

Cell culture and chemicals. AtT20 cells (mouse corticotroph tumor cell line) were provided by Shanghai Institute of Materia Medica (Shanghai, China) (29). AtT20 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen Life Technologies, Grand Island, NY, USA) at 37°C in 5% CO2. UA purchased from Sigma-Aldrich (St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) as a 100 mM stock solution and stored at -20°C. The cells were pretreated with JNK inhibitor SP600125 (Calbiochem, La Jolla, CA, USA), which was dissolved in DMSO 1 h prior to UA treatment.

2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium [Cell Counting kit-8 (CCK-8)] assay. Subsequent to being harvested and centrifuged, the AtT20 cells were resuspended in RPMI-1640 medium with 1% FBS and seeded in a 96-well plate at a density of 1 x 10^4/well. The cells were treated with different concentrations of UA for 24, 48 and 72 h, respectively, while only DMSO was added to the control wells. A total of 10 µl CCK-8 (Dojindo, Kumamoto, Japan) was added to each well and the microplate was incubated for 6 h at 37°C with 5% CO2. The optical density (OD) at 450 nm was read with a 96-well plate reader (Bio-Rad, Reinach, Switzerland). Experiments were conducted with five replicates.

Annexin V-fluorescein/propiodium iodide (PI) flow cytometric analysis. AtT20 cells were collected, centrifuged and washed with phosphate-buffered saline following treatment with the indicated amount of UA for 24 h with or without pretreatment of SP600125 (JNK inhibitor; Sigma-Aldrich) for 1 h followed by staining with Annexin V-fluorescein (FLOUS) Staining kit and PI (Roche Diagnostics, Mannheim, Germany) for 15 min at room temperature. For each example, 20,000 cells were analyzed on a flow cytometer (FACStar, BD Biosciences, Franklin Lanes, NJ, USA).

Assessment of caspase-3/-7, -8 and -9 activity. The caspase-3/7, -8 and -9 activities were measured by Caspase-Glo luminescent-based assays (Caspase-Glo kit; Promega, Madison, WI, USA). The cells were seeded with a total of 10^5 cells/well in a 96-well plate. Following treatment with UA (at various concentrations) in combination with SP600125 for 24 h, 100 µl of the Caspase-Glo-3/7, -8, -9 reagents were added to each well. The mixtures were incubated for 1 h and then transferred to a fluorescence microtiter plate. Quantification of luminescence was measured by a luminometer. To normalize the fluorescence intensity, the optical density values of the CCK-8 assay were measured by an Infinite F500 microplate reader (Tecan, Männedorf, Switzerland) as the viable cell number.

Assessment of ACTH by ELISA. The AtT20 cells were harvested, seeded in 24-well plates at a density of 2x10^5/500 µl/well and then added to different concentrations of UA for 48 h. The cell culture supernatants were collected and centrifuged. ACTH was assayed using a mouse ACTH ELISA kit (Uscn Life Science Inc., Wuhan, China) following the manufacturer's instructions.

Western blot analysis. Protein samples were extracted in radio-immunoprecipitation assay lysis buffer containing protease inhibitors and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Mitochondria were extracted using a mitochondrial isolation kit (Thermo Fisher Scientific). Following incubation for 10-30 min at 4°C, the supernatant was collected by centrifugation (10,000 x g, 15 min, 4°C). The cell lysates (20-40 µg) were detected using the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA) proteins were separated by 12% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA)
USA). Membranes were washed twice in Tris-buffered saline and Tween 20 (TBST) and incubated with blocking buffer (5% skimmed milk in TBST) for 60 min at room temperature. Next, the membranes were washed three times and incubated overnight at 4°C with primary antibodies. The membranes were incubated with secondary antibodies (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature. Following washing three times, the antibodies bound to the proteins were detected using enhanced chemiluminescence (Invitrogen Life Technologies). The following primary antibodies were used: anti-ACTH antibody (Ab) (1:1,000; Abcam); anti-phospho-p42/44 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204) Ab, anti-p42/44MAPK Ab, anti-phospho-p38 (Thr180/Tyr182) Ab, anti-p38 Ab, anti-phospho-JNK (Thr183/Tyr185) Ab, anti-JNK Ab, anti-B cell lymphoma 2 (Bcl-2) Ab, anti-Bcl-2-associated X (Bax) Ab, anti-β-actin Ab (all 1:1,000; Cell Signaling Technology, Inc.); anti-phospho-Bcl-2 (Ser70) Ab (1:1,000; Sigma-Aldrich); anti-cytochrome c Ab (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). First, the phospho-specific MAPK were detected. Next, the membranes were stripped and re-probed with anti-JNK, anti-extracellular signal-regulated protein kinases (ERK) and anti-p38 antibodies. The densities of phosphorylated MAPK bands were normalized to that of the total MAPK bands. The expression levels of p-Bcl-2 and total Bcl-2 were assessed in the same manner. The secondary antibodies were Goat anti-rabbit IgG.

Statistical analysis. The results were expressed as the mean ± standard deviation of three separate experiments. Data were analyzed by one-way analysis of variance followed by Fisher's least significant difference test or by Kruskal-Wallis test using SPSS 11 version software. P<0.05 was used to indicate a statistically significant difference.

Results

UA reduces viability and induces apoptosis of AtT20 cells. In order to investigate the effect of UA on cytotoxicity, the AtT20 cells were exposed to UA (10-100 µM) for 24, 48 and 72 h, respectively. The CCK-8 results revealed that UA inhibited the viability of AtT20 cells in a dose- and time-dependent manner (Fig. 1). The IC₅₀ value for 24 h was 20.02 µM. Considering the IC₅₀ for 24 h, the concentration of 20 µM was used for further analysis.

The apoptosis of AtT20 cells induced by UA was further evaluated. AtT20 cells were treated with UA (10, 20 and 40 µM) for 24 h, respectively. Afterwards, Annexin V-FLOUS/PI staining was performed and assessed by flow cytometry. The percentage of apoptotic cells, including early apoptotic cells (Annexin V-FLOUS+/PI⁻) and late apoptotic cells (Annexin V-FLOUS+/PI⁺) was significantly increased in a dose-dependent manner (Fig. 3A). In addition, the activity of caspase-3/7, which is the executioner of apoptosis, was examined. Caspase-3/7 activity was gradually enhanced from 2.8- to 13.7-fold as the dose of UA increased (Fig. 3D). These results confirm that cell viability loss induced by UA in AtT20 cells is facilitated by the induction of apoptosis.

UA decreases ACTH production and secretion in AtT20 cells. Next, it was investigated whether UA-induced apoptosis was accompanied with a decreased ACTH secretion. It has been reported that ACTH is derived from the POMC precursor peptide (30). In order to examine the effect on the intracellular levels of ACTH, UA (10, 20 and 40 µM) was added to AtT20 cells for 48 h, respectively, and the extracted total RNA was analyzed by qPCR. The results demonstrated that UA inhibited POMC mRNA (precursor of ACTH) in a dose-dependent manner. 40 µM UA achieved a maximum effect of inhibition (~0.3-fold compared with the control) (Fig. 2A). Western blot analysis also proved that UA decreased ACTH synthesis in a dose-dependent manner (Fig. 2B).

For determining the levels of ACTH, the conditioned medium was harvested and analyzed by ELISA. The data demonstrated that ACTH was reduced by UA in a dose-dependent manner. Compared with the control, ACTH levels were 0.62-, 0.48-, 0.3-fold, respectively (Fig. 2C). These results indicated that UA decreased the expression of POMC mRNA as well as ACTH synthesis and secretion in AtT20 cells in a dose-dependent manner.
Endogenous and exogenous signaling pathways are involved in UA-induced apoptosis. Apoptosis is mediated by endogenous and exogenous signal transduction. To address whether the two pathways were involved in UA-induced apoptosis, caspase-8, -9, cytochrome c, Bcl-2 and Bax were assayed. AtT20 cells were treated with UA (10, 20 and 40 µM) for 24 h. Caspase-8 activity increased by 1.35-, 4.93-, 7.5-fold, respectively, as compared with the control (Fig. 3B). The levels of Bcl-2 and cytochrome c in mitochondria were reduced while cytochrome c in cytosol was increased along with increasing dose of UA (Fig. 3E and F). The protein levels of Bax were normal while the ratio of Bcl-2/Bax decreased (Fig. 3E and G). Caspase-9 was also significantly increased by 4.27-fold subsequent to treatment with 40 µM UA (Fig. 3C). The results revealed that endogenous and exogenous signaling pathways contributed to UA-induced apoptosis.
The JNK pathway is involved in UA-induced apoptosis. Cell proliferation and apoptosis, and the MAPK family are closely associated (31). UA has been shown to activate the MAPK signaling pathway in various cells (23,32). To further investigate the exact molecular mechanism of UA-induced apoptosis and identify whether the MAPK signaling pathway was also activated by UA in AtT20 cells, phosphorylation of JNK, ERK1/2 and p38 was detected. The results revealed that 20 µM UA upregulated the expression of phosphorylation of JNK, which reached a maximum from 1 to 3 h, but...
had no effect on the phosphorylation of ERK-1/2 and p38 (Fig. 4A and B). To further confirm whether JNK activation mediated UA-induced apoptosis, AtT20 cells were pretreated with 10 μM SP600125 (JNK inhibitor) for 1 h followed by 20 μM UA for 24 h (Fig. 4C). The percentages of early and late apoptotic cells were 19.6 and 10.6% respectively, which were significantly less compared with that of cells in the group treated with 20 μM UA only (Fig. 3A).

It has been reported that JNK activation inactivated Bcl-2 by increasing phosphorylation of Bcl-2 (33,34). The results showed that 20 μM UA treatment for 3 h increased phosphorylated-Bcl-2 (Ser 70), while inhibition of JNK activation by SP600125 could downregulate the phosphorylation (Fig. 5A), demonstrating that JNK activation was involved in the upregulation of UA-induced Bcl-2 phosphorylation and resulting apoptosis.

To evaluate whether the activation of caspases was affected by Bcl-2, caspase-9 and -3/7 were assessed. The results revealed that caspase-3/7 and -9 activation were partly blocked in the UA group in the presence of SP600125 (Fig. 5A-C). Thus, the data indicated that JNK activation was involved in the exogenous signaling pathway in UA-induced apoptosis by increasing phosphorylated Bcl-2.

Discussion

Pituitary adenoma accounts for a significant proportion of primary intracranial tumors. Although ACTH-producing pituitary adenoma is benign, it does cause detrimental effects due to excessive hormone secretion resulting in significant mortality and an impaired quality of life. Removing or shrinking the tumor and normalization of ACTH excess are the crucial goals of treatment. Thus far, no reliable medical therapies exist to directly target the pituitary tumor growth and ACTH secretion. In the present study, it was demonstrated that UA could function as a potential novel and potent therapeutic agent targeting directly on ACTH-producing pituitary adenomas.

The present study revealed that UA was able to reduce the viability and induce apoptosis of AtT20 cells (mouse corticotroph tumor cell line) in a dose-dependent manner. The results were in consistency with other studies in various tumor types (21,35-39), which indicated that UA had a pro-apoptotic effect on ACTH-producing pituitary tumor cells.

Excess ACTH levels and hypercortisolism may result in high co-morbidity and mortality. Normalization of hormone excess is the therapeutic goal of treatment. Numerous compounds, including dopamine agonists, thiazolidinediones and curcumin have been reported to inhibit ACTH synthesis and (or) secretion (12,40,41). However, none of these agents has been proven to be effective in the management of ACTH-producing pituitary adenoma. In the present study, it was identified that UA decreased ACTH production and secretion in AtT20 cells in a dose-dependent manner, demonstrating the potential of UA to be a novel agent for the management of ACTH-producing pituitary adenoma.

Apoptosis has an essential role in tumorigenesis. Two major apoptotic pathways have been identified (42). One is the exogenous pathway, which involves the binding of a ligand to the death receptor and subsequent caspase-8 activation (43). The other is the endogenous pathway, relying on the release of cytochrome c from mitochondria to the cytosol which recruits the initiator pro-caspase-9, which yields activated caspase-9 and finally activates caspase-3 (44). The key components of the mechanism involved in mitochondria-dependent apoptosis are the Bcl-2 family of proteins, including pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins (45). Bcl-2 proteins usually form heterodimers with Bax, resulting in the release of cytochrome c from mitochondria to the cytosol and triggering the death program. In the present study, it was revealed that UA increased caspase-9 and -8 activities, decreased the ratio of Bcl-2/Bax and promoted cytochrome c release from mitochondria in a dose-dependent manner. The results indicated that exogenous and endogenous pathways were involved in UA-induced apoptosis in AtT20 cells.

To elucidate how UA triggered the apoptotic process, the exact molecular mechanisms were further investigated. It has been reported that the MAPK pathway had a vital role in UA-induced apoptosis in various tumor cells (32,37,46). In the present study, UA was found to increase the phosphorylation of JNK, but not ERK1/2 or p38, in a time-dependent manner. Pretreatment with JNK inhibitor SP600125 blocked UA-induced cleavage of caspase-3 and -9. Previous studies have reported that the JNK pathway participated in UA-induced the apoptotic signaling pathway via controlling phosphorylation of Bcl-2 (36,37,47). The phosphorylation of Bcl-2 resulted in the degradation of Bcl-2, which led to the release of Bax from the Bcl-2/Bax heterodimer and triggered apoptosis (48). The present study demonstrated that UA treatment for 24 h decreased the levels of Bcl-2 and additionally induced the phosphorylation of Bcl-2 in AtT20 cells. Furthermore, pretreatment with SP600125 was able to partly block UA-induced Bcl-2 phosphorylation (Ser70) and degradation. These findings revealed that UA-induced JNK activation may promote Bcl-2 phosphorylation, degradation and finally induce apoptosis.

In conclusion, the present study demonstrated that UA inhibited viability, induced apoptosis and decreased ACTH production in AtT20 cells. The induction of apoptosis involved exogenous and endogenous pathways. Increased phosphorylation of Bcl-2 via JNK activation had a crucial role in UA-induced apoptosis in AtT20 cells. These findings indicate the potential of UA as a novel potential therapeutic agent targeting ACTH-producing pituitary adenoma. Further clinical studies are required to examine the efficacy and safety of UA.

Acknowledgements

The abstract of this study has been published in Endocr Rev Vol. 34, (03 Meeting Abstracts): Sun-188, 2013.

References


