Original Ariticle
Expression of apolipoprotein D and androgen receptor in axillary osmidrosis and its molecular mechanism

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Abstract: Objective: To investigate the expression of apolipoprotein D (ApoD) and androgen receptor (AR), two proteins related to E-3M2H secretion, in the apocrine sweat gland of patients with axillary osmidrosis (AO) and healthy subjects, and to explore the cause of abnormal ApoD expression in these patients. Methods: Samples were collected from healthy controls (n=4) and AO patients (n=10). Immunohistochemistry, real-time PCR and western blot assay were performed to measure the mRNA and protein expression of ApoD and AR. In vitro sweat gland cells were treated with androgen to explore the AR signals in regulation of ApoD expression and the role of JNK1 signaling pathway in the ApoD expression. Results: There was significant difference in the expression of ApoD and AR between AO patients and healthy controls. The ApoD expression in AO patients was 2-fold higher than that in healthy controls and the AR expression in AO patients was also markedly increased when compared with healthy controls. Moreover, the activation of JNK1 increased in AO patients. Androgen can increase the ApoD expression in healthy subjects accompanied bu JNK1 activation. Inhibition of JNK1 activation may reduce the ApoD expression in AO patients and the androgen induced ApopD expression. Conclusion: The increase ApoD expression is closely related to the AR signaling pathway. JNK1 activation is a major cause of increased ApoD expression in AO patients and the androgen induced ApopD expression. To inhibit the JNK1 activation may suppress the endogenous ApoD expression in AO patients and the androgen induced ApopD expression.

Keywords: Axillary osmidrosis, apolipoprotein D, c-Jun N-terminal kinase, expression regulation

Introduction

Axillary osmidrosis (AO) is a common disease in the Department of Plastic and Reconstructive Surgery. AO psychologically affects these patients and influences their daily life and work. To date, some strategies have been developed for the treatment of AO, and the therapeutic efficacy varies. A majority of these stategies are invasive and usually have risk for complications. To elucidate the pathogenesis and pathophysiology of AO is beneficial for developing novel non-invasive strategy for the treatment of AO. There is evidence showing that (E)-3-methyl-2-hexenoic acid (E-3M2H) plays an important role in the pathogenesis of AO, and the apolipoprotein D (ApoD) can regulate the E-3M2H secretion. However, the ApoD expression and its relation with AO are still unclear in AO patient. To investigate the expression of

ApoD and the underlying mechanism is crucial for understanding the pathogenesis of AO. In the present study, the expression of AR and ApoD and its relation with AO were investigated, and the regulatory effect of AR signal, especially the JNK1 signaling pathway, on ApoD expression in the apocrine sweat gland was explored.

Materials and methods

Sample collection

Male patients (n=10) with AO were recruited from the Department of Plastic and Reconstructive Surgery of Tangdu Hospital of Forth Military Medical University from October 2009 to May 2010 and 4 male subjects receiving surgery for scar repair or others served as controls. The fresh axillary skin containing adipose tissues (about 6×2×2 cm) of both sides was collected for experiments.

Cell culture

The skin was washed with D-Hanks solution and the adipose tissues were removed. The skin was then cut into blocks (1-mm^3) which were digested in type II colagenase for 1 h in an incubator. One day later, the sweat gland was collected under a light microscope and transferred into a flask for cultre. When the sweat gland was adherent to the flask wall, culture continued in 2-ml of medium which was refreshed every 2-3 days. Generally, the sweat gland is contaminated with fibroblasts which are difficult to be adherent to the flask wall. For purification of sweat gland cells, digestion was performed with trypsin. Following digestion, fibroblasts were shedding firstly and then removed by aspiration. Digestion continued and the sweat gland cells were harvested with high purify. Cells were maintained in DMEM containing 10% FBS.

Treatment with JNK inhibitor

The cells from AO patients were seeded into 6-well plates and maintained overnight. When the cell confluence reached 70%, cells were treated with JNK inhibitor at 10^{-6} M. Following culture for 24 h, the cells were harvested for further detection.

Treatment with 5α-DHT

The cells from controls were seeded into 6-well plates and maintained overnight. When the cell confluence reached 70%, cells were treated with 5α-DHT at 10^{-7} M and 10^{-6} M. In addition, for cells treated with 5α-DHT at 10^{-6} M, JNK inhibitor was also administered at 10^{-6} M, followed by culture for 24 h. The cells were harvested for further experiment.

Western blotting

AO tissue proteins were extracted using ice-cold lysis buffer containing a protease inhibitor cocktail (Roche), and the proteins in the supernatant were quantified using the bicinchoninic acid method (Pierce, Rockford, IL). Fifty micrograms of protein were separated per lane by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoretic transfer of the proteins to a polyvinylidene difluoride membrane (Millipore, Bedfordshire, UK) using a MiniProtein III system (Bio-Rad), immunoblotting was performed using rabbit polyclonal primary anti-ApoD or AR antibody (1:2000, Abcam, Cambridge, UK) and primary anti-β-actin (diluted 1:4000, Santa Cruz Biotechnologies, Santa Cruz, CA), and developed in an enhanced chemiluminescence system (Pierce) using specific peroxidase-conjugated anti-IgG secondary antibodies (1:4000, Santa Cruz). For quantification purposes, densitometric measurements were performed using the Quantity One image analysis software for Windows (Bio-Rad). All ApoD and AR values were normalized to β-actin levels.

Immunohistochemistry for ApoD and AR

Paraffin sections were cut and mounted on glass slides, and 5 μm sections from formalin-fixed and paraffin-embedded specimens were deparaffinized using xylene and rehydrated in graded ethanol. Samples were then preincubated with 3% H_2O_2 to eliminate endogenous peroxidase activity. Antigen retrieval was achieved by heating the sections (for 2 min to 100°C) in citric acid buffer (0.01 mol/L, pH 6.0). Sections were incubated at 4°C overnight in a 1:800 dilution of the rabbit polyclonal primary anti-ApoD or AR antibody (Abcam). After three washes, goat anti-rabbit horseradish peroxidase-conjugated antibody (Envision detection kit, Gene-Tech, Shanghai, China) was applied for one hour. Sections were developed using a peroxidase substrate DAB kit (Gene-Tech) and counterstained using hematoxylin. Sections were subsequently dehydrolyzed before they were mounted on coverslips. Images were collected using an Eclipse TE2000-U microscope system (Nikon, Tokyo, Japan). The primary antibody was replaced with phosphate-buffered saline in the control experiments.

Detection of ApoD mRNA expression by RT-PCR

Total tissue RNA was extracted using Trizol reagent (Invitrogen), and cDNA was synthesized from total RNA using oligodT primers (Invitrogen). RNA integrity was confirmed by electrophoresis on 1% agarose gels stained with ethidium bromide. Quantitative reverse-transcription polymerase chain reaction (PCR) was utilized to compare the relative amounts of ApoD in the AO tissues and was carried out on a Bio-Rad System (CFX96). For each reaction,
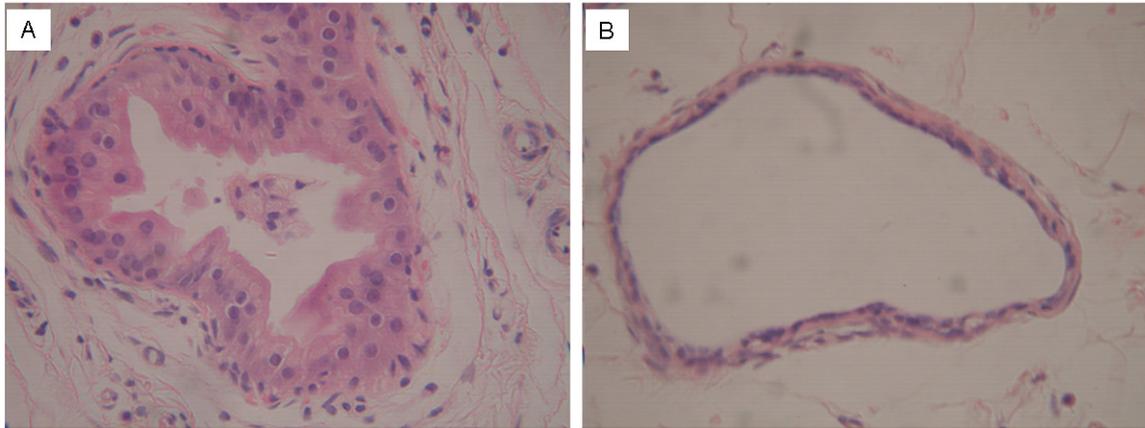
Figure 1. Apocrine sweat gland in active phase in AO group (A) and control group (B).
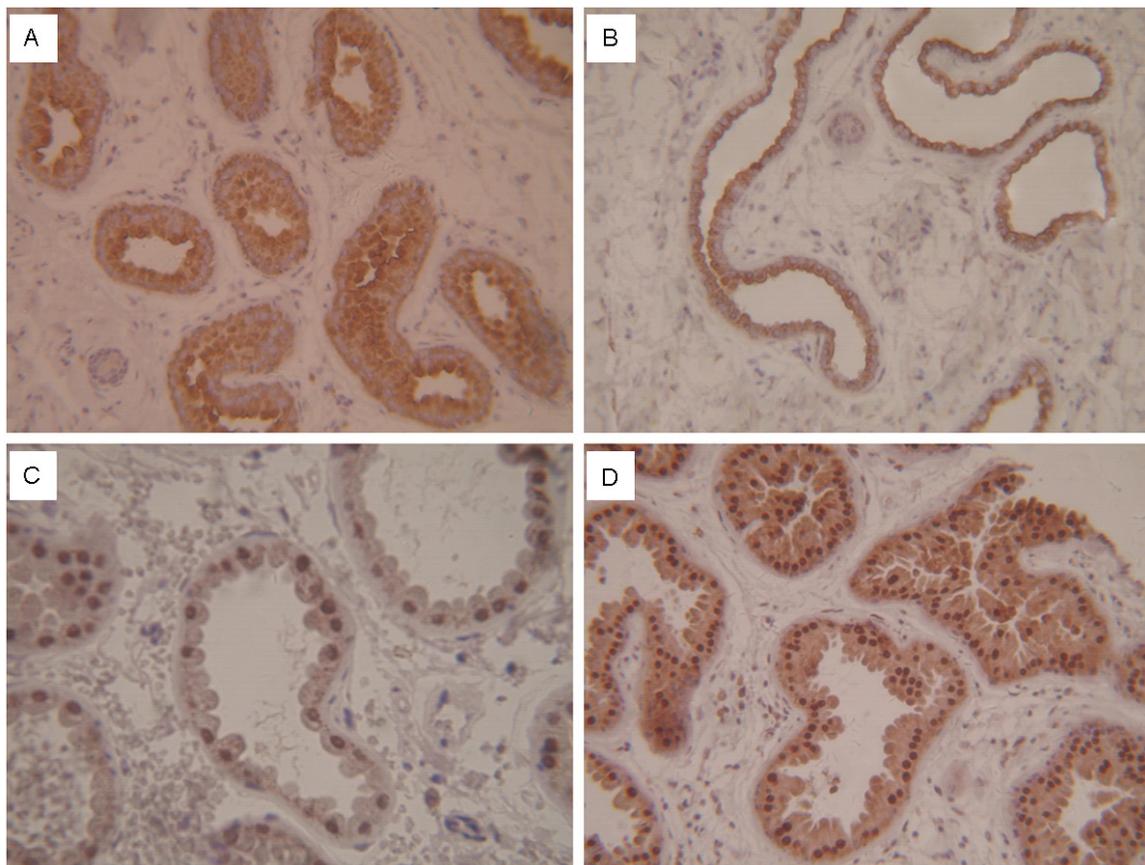
Figure 2. A. High ApoD expression in apocrine sweat gland cells of AO group; B. Low ApoD expression in apocrine sweat gland cells of control group; C. Low AR expression in nucleus of apocrine sweat gland cells of control group; D. High AR expression in nucleus of apocrine sweat gland cells of AO group.

cDNA was added to 20 µL of reaction mixture containing 10 µL of SYBR Green PCR Master Mix (Toyobo, Japan) and 300 nM primers (Invitrogen). For quantification, the target sequence was normalized to GAPDH mRNA levels. PCR was performed under the following conditions: 95°C for 30 s, 57°C for 15 s, and 72°C for 30 s for 40 cycles. Heating for 2 min at
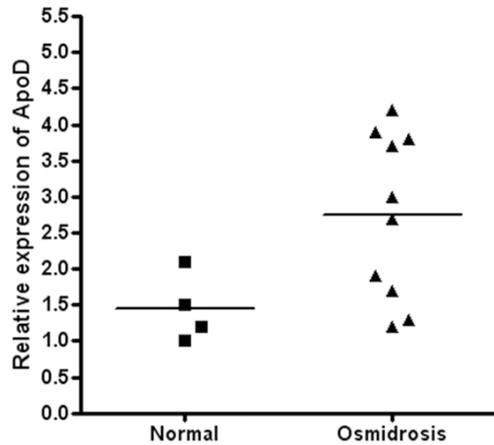
Figure 3. ApoD mRNA expression increased in AO patients.

Figure 4. Increased AR and ApoD expression accompanied by JNK1 activation in AO patients.

95°C preceded the cycles. The results were quantified using the comparative CT method. PCR products were analyzed by electrophoresis with 1.5% agarose gel and visualized by ethidium bromide staining. Primer sequences used for amplification were as follows: ApoD (product size 152 bp) upstream primer, 5'-taaacat-cagagacctgaag-3'; downstream primer, 5'- agaa-tcagccgatttgagat-3'; GAPDH (product size 200 bp) upstream primer, 5'-aggctgtgggcaaggt-catc-3'; downstream primer, 5'-accactgacac-gttggcagt-3'.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0. Quantitative data were expressed as mean ± standard deviation ($\bar{x}$ ±s) and t test was applied for comparisons. A value of P<0.05 was considered statistically significant.

Results

Morphology of apocrine sweat gland

The secretory activity of apocrine sweat gland may switch between the active phase and the quiescent phase depending on the in vivo and in vitro environment. In the present study, the morphology of apocrine sweat gland was observed. Results showed the apocrine sweat gland at different stages was observed in the AO group and control group, but the number of apocrine sweat gland in the AO group was markedly larger than that in the control group, and the apocrine sweat gland was even absent in

severe sections of the control group. The apocrine sweat gland in active phase had small lumen, sweat gland cells were columnar, and the top of these cells protruded into the lumen to form cat-like protuberance. Some cells shedded into the center of lumen (**Figure 1A**). The apocrine sweat gland in quiescent phase had large lumen, cells were flat and had no protuberance (**Figure 1B**).

Immunohistochemistry for ApoD and AR

In the AO group, numerous brown granules were found in the apocrine sweat gland cells, and these cells were strong positive for ApoD (**Figure 2A**). In the control group, only a few brown granules were noted in the apocrine sweat gland cells which were weakly positive for ApoD (**Figure 2B**). The ApoD expression in the apocrine sweat gland cells of AO group was markedly higher than that in the control group. In the control group, only a few brown granules were found in the nucleus of apocrine sweat gland cells which were weakly positive for AR (**Figure 2C**). In the AOP group, a large amount of brown granules were noted in the nucleus of apocrine sweat gland cells which were strongly positive for AR (**Figure 2D**).

ApoD mRNA expression by real-time PCR

Samples were collected from 10 patients with AO and 4 healthy controls, and the ApoD mRNA expression was detected. Results showed the ApoD mRNA expression was at a low level in
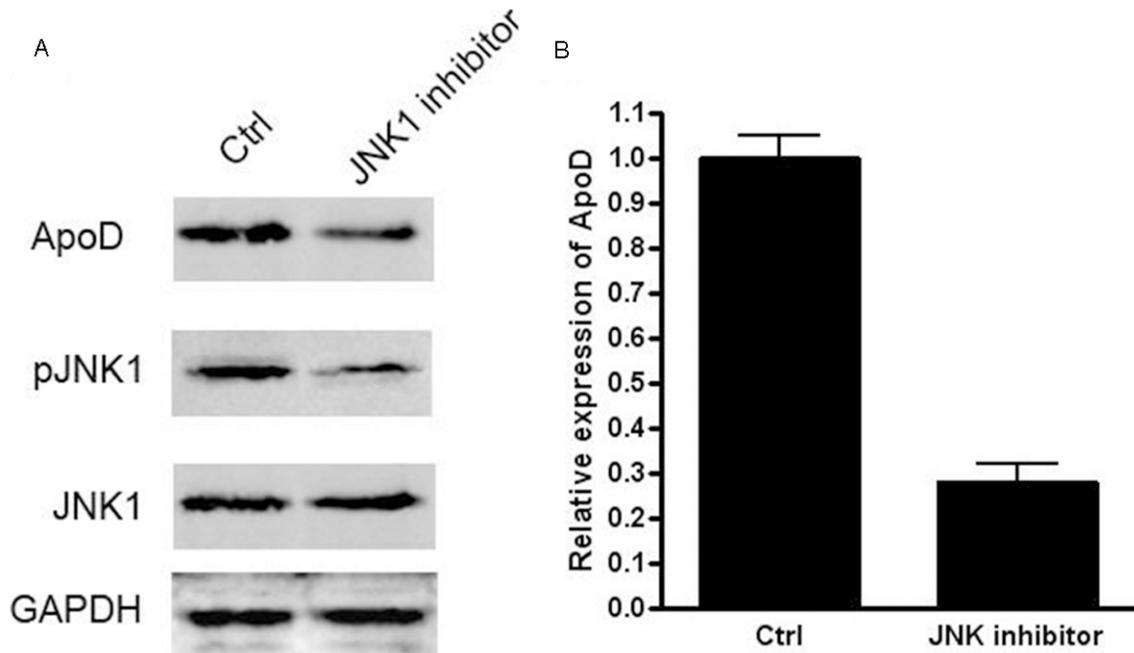
Figure 5. Inhibition of JNK1 phosphorylation significantly suppresses the protein (A) and Mrna (B) expression of ApoD in AO patients.
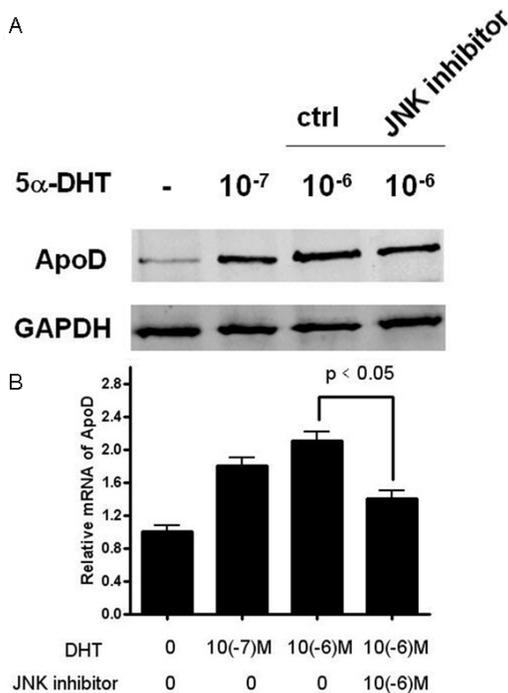
Figure 6. 5α-DHT increases ApoD protein (A) and Mrna (B) expression in a JNK1 dependent manner.

higher than that in healthy controls (**Figure 3**) showing significant difference between two groups.

Increase in JNK1 activation in AO patients

The protein expression of ApoD was detected in these 14 patients. In addition, the expression and activation of JNK1 were also detected in these patients. Results showed there was no difference in the JNK1 expression between AO patients and controls, but the expression of phosphorylated JNK1 was markedly increased in AO patients (**Figure 4**). This suggests that increased JNK1 phosphorylation may be an important molecular event in the pathogenesis of AO.

On the basis of above findings, the ApoD expression was measured following JNK1 inhibition. Cells were seeded into 6-well plates and treated with JNK1 inhibitor (SP600125). Results showed JNK1 inhibitor could effectively inhibit the JNK1 phosphorylation and the ApoD expression (**Figure 5A**). This suggests that JNK1 activation is an important cause of increased ApoD expression. To elucidate that JNK1 can regulate the protein or mRNA expression of ApoD expression, the mRNA expression

controls but at a high level in AO patients. The ApoD expression in AO patients was 2 folds

of ApoD was also measured. Results showed JNK1 inhibition could effectively suppress the mRNA expression of ApoD (**Figure 5B**). This suggests that JNK1 may affect the ApoD expression at the mRNA level. The previous findings suggest that AR can regulate the JNK1 activation, and JNK1 can also promote the transcription of AR.

In this study, we further investigated whether AR directly affects the regulation of ApoD expression by JNK1. The apocrine sweat gland cells from controls were treated with 5α-DHT at 10^{-7} M and 10^{-6} M for 24 h. Results showed 5α-DHT could increase the ApoD expression in a concentration dependent manner, but JNK1 inhibit the ApoD expression to a certain extent (**Figure 6A**). All these findings suggest that AR can regulate the ApoD expression in JNK1 dependent and independent manners. At mRNA level, similar results were found in ApoD expression (**Figure 6B**). 5α-DHT increased the mRNA expression of ApoD in a concentration dependent manner, but JNK1 inhibited the ApoD expression to a certain extent.

Discussion

AO is a common familial hereditary disease and its prevelance is about 4.56% in Han Chinese [1-2]. In recent years, studies have shown that the abnormal secretion of aprocrine sweat gland involves in the pathogenesis of AO, and the aprocrine sweat gland plays an important role in the occurrence of AO [3]. There is evidence [4] showing that the aprocrine secretion odor-binding protein (ASOB) is related to the secretion of E-3M2H. The major component of terrible odor E-3M2H, and others include volatile sulfur compounds [5]. In addition, sex hormone has been found to regulate the AO [6] which also explains that AO is frequently found in patients in pubertal stage. Kurata et al found that axillary apocrine sweat gland was a target of AR [7]. Beier et al [6] proposed that there was AR in the nucleus of apocrine sweat gland cells, which was comparable between females and males. There are two subtypes of ASOB in the apocrine sweat gland of humans: ASOB1 and ASOB2 [8], and ASOB2 plays a dominant role in the production of odor in AO [9]. In the cytoplasm, E-3M2H can bind to the N terminal of ASOB2 via the covalent bonds forming a complex. This complex is then transported to the surface of skin and degraded into E-3M2H, HMHA and Gln by bacteria resulting in the production of terrible smell. In addition, study also reveals that the apocrine sweat gland cells can produce the terrible odor in the absence of bacteria [10]. There is complicated regulation between AR and ASOB, which is crucial for the pathogenesis of AO. In our previous study, the relationship between the expression of AR and ASOB in the apocrine sweat gland cells and the pathogenesis of AO was investigated at morphological and protein levels, and this relation is required to be confirmed at gene level. Increased secretion of E-3M2H is an important basis for the occurrence of AO, and ApoD is a pivotal factor regulating the E-3M2H secretion.

On the above findings, the secretion of E-3M2H is an initiator of production of terrible odor, and the abnormal regulation of ApoD expression is a major cause of AO. Sex hormone can regulate the ApoD expression via corresponding receptor involving in the physiology and pathophysiology. To date, the role of hormone and hormone receptor in the occurrence and development of AO has not been fully elucidated. In recent studies on prostate cancer, results showed AR could regulate the ApoD expression [11], which suggests that AR involves in the regulation of ApoD expression in the occurrence of AO.

Our results showed AR could stimulate the ApoD expression via JNK1, but inhibition of JNK1 failed to completely block the AR related up-regulation of ApoD. This suggests that AR can also stimulate the ApoD expression in a JNK1 independent manner. In addition, JNK1 can stimulate the transcription of AR, suggesting that JNK1 can regulate the ApoD expression via modulating the AR signaling pathway. We speclate that there is a feedback loop between the AR signaling pathway and the JNK1 signaling pathway. Of note, the JNK1 activation is regulated by hormone, and some in vivo stimuli can promote the JNK1 activation. In the AO patients, whethere other factors also involve in the increased JNK1 activation is required to be further investigated.

Taken together, our findings demonstrate that AR can promote the ApoD expression via activating JNK1. To further investigate the role of JNK1 activation and Apo expression in the

pathogenesis of AO is helpful to understand the pathogenesis of AO and develop the non-invasive strategies for the treatment of AO.

Disclosure of conflict of interest

None declared.

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