

Activating Transcription Factor 3 (ATF3) in the Human Adrenal Cortex: Its Possible Involvement in Aldosterone Biosynthesis

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The activating transcription factor 3 (ATF3) is a member of the cAMP-responsive element-binding (CREB) protein family of transcription factors. ATF3 is expressed in H295R human adrenocortical carcinoma cells and considered a rapid-responder gene to angiotensin-II stimulation. However, the functions of ATF3 in human adrenocortical tissues have remained unknown. In this study, we analyzed the localization and possible regulatory mechanisms of ATF3 in human adrenocortical cells and tissues. The expression levels of ATF3 mRNA were analyzed in 66 aldosterone-producing adenomas (APA) and 14 cortisol-producing adenomas (CPA) using real-time RT-PCR. To localize the ATF3 protein, we performed immunohistochemical analysis in 20 non-pathological adrenal glands, 9 adrenal glands with idiopathic hyperaldosteronism (IHA), 20 APA, and 5 CPA using a mouse monoclonal antibody against human ATF3. We showed that ATF3 mRNA levels were higher in APA compared to CPA ($P = 0.0053$). ATF3 was immunolocalized to the zona glomerulosa of non-pathological adrenal glands and adrenal glands with IHA, and diffusely detected in the tumor cells of APA and CPA. Subsequently, H295R cells were treated for 6 h with each inhibitor of Src kinase (SRC), PKC, JAK2, and calcium-dependent calmodulin kinase-II (CaMKII) in the presence or absence of angiotensin-II. The expression levels of ATF3 mRNA were increased by angiotensin-II (about 3.5-fold induction), but the magnitude of the induction was significantly decreased in the presence of an inhibitor for SRC (PP2) or CaMKII (KN93). These results suggest that ATF3 is a downstream target of SRC and CaMKII signaling, and may be involved in adrenocortical aldosterone synthesis.

Keywords: adrenal cortex; adrenocortical adenoma; cell signaling pathways; immunohistochemistry; zona glomerulosa

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Introduction

The activating transcription factor 3 (ATF3) is a member of the cAMP-responsive element-binding (CREB) protein family of transcription factors (Hai et al. 1999). Some of these transcription factors can regulate gene expression by binding to the consensus ATF/CREB cis-regulatory element via a basic-region leucine zipper domain (Hai et al. 1999). However, ATF3 has been suggested to be a common stress response mediator that can be rapidly induced by a broad spectrum of cellular stress stimuli, including DNA damage, cell injury, oxidative stress, and endoplasmic reticulum stress, a characteristic that makes it distinct from other ATF/CREB members (Hai and Hartman 2001). Because of this apparent induction by cellular stress, it has been pro-

posed that ATF3 can play some role in the maintenance of cell integrity and homeostasis under stressful conditions (Hai et al. 1999, 2010).

Therefore, ATF3 has been generally considered as a stress inducible immediate early gene and an adaptive response gene (Thompson et al. 2009; Zmuda et al. 2010), and it appears to play an important role in mediating the cellular response to injury and stress (Hai et al. 2010). Nevertheless, it has also been suggested that ATF3 can enable cells to cope with various intracellular and extracellular changes, which are not directly harmful (Hai et al. 2010). For instance, most tissues express very low levels of ATF3 under physiological conditions (Hsu et al. 1991; Chen et al. 1994; Allen-Jennings et al. 2001).

Recently, ATF3 has been reported as an early response

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gene in adrenocortical cells, including bovine and rat zona glomerulosa cells, and the human adrenocortical carcinoma cell line H295R (Nogueira et al. 2009). Nogueira et al. (2009) demonstrated that the mRNA levels of ATF3 were elevated up to 2.5 fold over controls after 1 h of angiotensin-II (AII) stimulation in H295R cells. However, the details of ATF3 expression in human adrenocortical tissues, as well as the mechanisms that may regulate this expression have remained unknown.

In our present study, we immunolocalized the ATF3 protein in human adrenocortical tissues, and evaluated its mRNA levels in steroid-producing adrenocortical tumors. In order to investigate possible intracellular pathways that involve ATF3, we performed a series of experiments using the H295R adrenocortical cells.

Materials and Methods

Human adrenal cases

The research protocols in this study were approved by the ethics committees at Tohoku University Graduate School of Medicine (Sendai, Japan) and the Fukushima Medical University (Fukushima, Japan).

For the real-time RT-PCR analysis (qPCR), adrenocortical neoplasm specimens were obtained from Tohoku University Hospital [60 aldosterone-producing adenomas (APA) and 8 cortisol-producing adenomas (CPA)] and Fukushima Medical University Hospital (6 APA and 6 CPA).

For the immunohistochemical analysis, we retrieved 20 non-pathological adrenals (NA), 9 adrenal glands with idiopathic hyperaldosteronism (IHA), 20 APA and 5 CPA cases from the surgical pathology files of Tohoku University Hospital (Sendai, Japan). NA tissues were obtained from nephrectomy cases due to renal carcinoma, and were subsequently evaluated to confirm the absence of neoplastic invasion, necrosis or other histopathological abnormalities.

RNA isolation and real-time RT-PCR (qPCR)

Isolation of RNA and subsequent cDNA synthesis and qPCR analysis were performed as previously reported (Felizola et al. 2014c, d). The primer sequences used in our study were: *ATF3* forward 5'-CAG GGT TGT GCT TTC TAG CAA ATA TGC TG-3', reverse 5'-AAG TCA CAT CAG TGT TGT GTG ACA CAA AC-3'; and *RPL13A* forward 5'-CCT GGA GGA GAA GAG GAA AG-3', reverse 5'-TTG AGG ACC TCT GTG TAT TT-3'. The cDNA produced from a human brain specimen was used as a positive control in the qPCR experiments. A house-keeping gene *RPL13A*, referent to the 60S ribosomal protein L13a, was used as an internal control.

When tissue samples were analyzed, the relative gene expression was calculated as previously reported (Felizola et al. 2014d). To analyze the data derived from cell experiments, the relative gene expression was calculated by the $\Delta\Delta C_t$ method as previously reported (Nogueira et al. 2007).

Immunohistochemical analysis

A mouse monoclonal antibody against human ATF3 was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used at a 1:200 dilution. The immunohistochemical technique was performed as previously described (Felizola et al. 2014c, d).

Cell culture

H295R human adrenocortical carcinoma cells (Bird et al. 1995) were cultured in DMEM/Eagle's F12 medium (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% Cosmic Calf Serum (Hyclone laboratories Inc., Nampa, ID, USA), 1% penicillin/streptomycin (Invitrogen), and 0.01% gentamycin (Sigma-Aldrich). Cells were maintained at 37°C under humidified atmosphere with 5% CO₂.

Treatment of H295R cells with angiotensin-II and forskolin in a time-dependent manner

H295R cells were transferred to 12-well dishes at 600,000 cells per well, and treated with AII or forskolin (FSK) as reported (Felizola et al. 2014c). RNA was extracted at 3, 6, 12, and 24 h time points following treatment (RNeasy Mini Kit, QIAGEN, Hilden, Germany).

Inhibition of intracellular pathways in the presence or absence of AII

H295R cells were transferred to 12-well dishes, and treated with each of the following substances for 6 h: PP2 (inhibitor of Src kinase (SRC)), Go6983 (inhibitor of protein kinase C (PKC)), NSC33994 (inhibitor of Janus kinase 2 (JAK2)), and KN93 (inhibitor of calcium-dependent calmodulin kinase-II (CaMKII)). The treatment of H295R cells was conducted either in the presence or the absence of AII (10 nM). Independent triplicate repeats were performed for all experiments.

Statistical analysis

Human tissue qPCR data were evaluated using Mann-Whitney tests with the significance level set to $\alpha = 0.05$.

qPCR data from the cell experiments were evaluated in groups of 3 (basal, AII, FSK) or 4 (basal, inhibitor, AII, AII + inhibitor) using Mann-Whitney multiple comparison tests with the significance level set to $\alpha = 0.05$. The Bonferroni inequality was used to correct multiple comparisons, with $0.05/3 = 0.016$, resulting in $P < 0.016$, or $0.05/6 = 0.0083$, resulting in $P < 0.0083$ as indicating statistical significance, respective to the number of group variants.

Results

qPCR analysis of human adrenocortical tissues

The mRNA levels of ATF3 were significantly higher in APA compared to CPA cases ($P = 0.0053$), as illustrated in Fig. 1.

Immunohistochemical analysis of human adrenocortical tissues

ATF3 immunoreactivity was markedly detected in the zona glomerulosa of NA and IHA, while virtually absent in the zona fasciculata (Fig. 2A, B) and the zona reticularis (not shown).

ATF3 immunoreactivity was diffusely detected in APA (Fig. 2C), while detected either diffusely or focally in CPA cases examined (Fig. 2D).

qPCR analysis of H295R cells time-course experiment

The treatment with AII or FSK elevated the mRNA levels of ATF3 in H295R cells and reached a peak by 6 h, and the expression levels of ATF3 mRNA abruptly decreased to near basal levels from 12 h (Fig. 3).

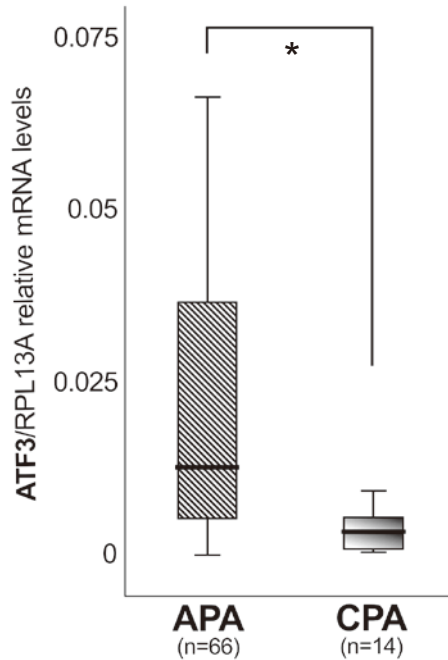


Fig. 1. ATF3 mRNA levels in aldosterone-producing adenoma (APA) and cortisol-producing adenoma (CPA). The mRNA levels of ATF3 were significantly higher in APA compared to CPA. * $P = 0.0053$. The vertical whiskers of the box plots represent the variability outside the upper and lower quartiles, and the bands inside the boxes represent the medians.

Analysis of ATF3 mRNA levels after treatment with intracellular pathway inhibitors

The SRC inhibitor (PP2) and the CaMKII inhibitor (KN93) caused a statistically significant decrease in ATF3 mRNA levels when treated in combination with AII treatment, compared to AII alone ($P = 0.0002$ for PP2 + AII vs. AII; and $P < 0.0001$ for KN93 + AII vs. AII). However, none of the inhibitors examined in this study exerted any significant changes to basal levels of ATF3 in the absence of AII (Fig. 4).

Discussion

In our results, we detected higher expression levels of ATF3 mRNA in APA compared to CPA, and the ATF3 protein was immunolocalized in the zona glomerulosa of NA and adrenals with IHA, while diffusely immunoreactive in the tumor cells of adrenocortical adenomas. In addition, results from our *in vitro* experiments show that the mRNA levels of ATF3 can be influenced by the inhibition of intracellular pathways related to aldosterone biosynthesis (Nogueira et al. 2009; Nakamura et al. 2014) in H295R cells.

It was previously proposed that AII could stimulate intracellular pathways involving SRC, PKC, JAK2, and CaMKII (Nogueira et al. 2009; Nakamura et al. 2014). Results of our study show that the mRNA levels of ATF3

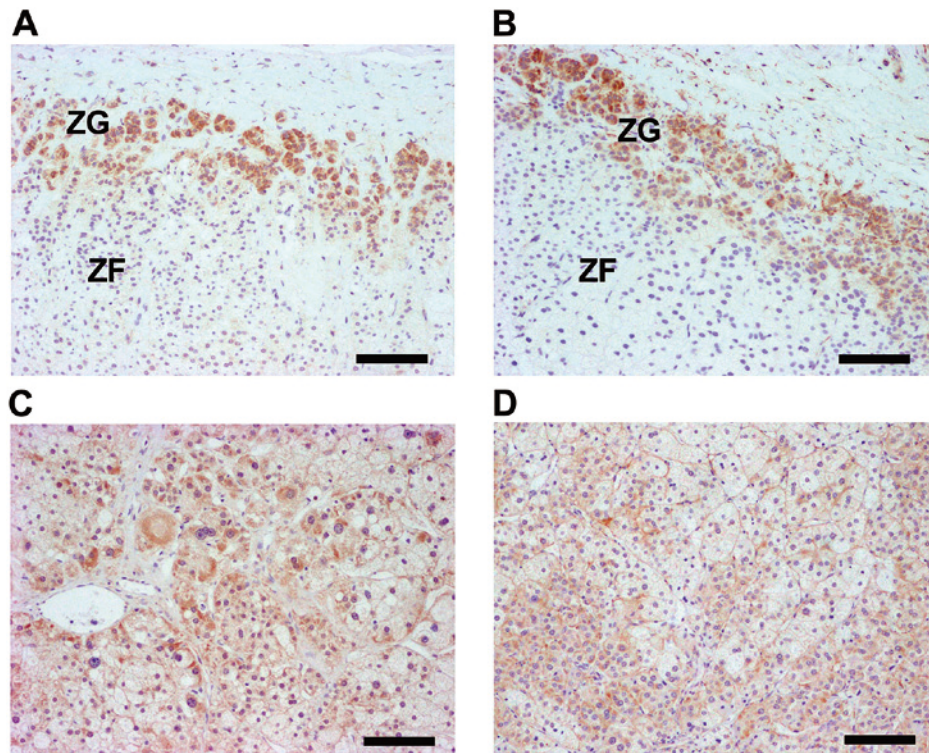


Fig. 2. Immunohistochemical localization of ATF3 in human adrenocortical tissues. Marked ATF3 immunoreactivity was detected in the zona glomerulosa (ZG) of [A] the normal adrenal gland (NA; $n = 20$) and of [B] idiopathic hyperaldosteronism (IHA; $n = 9$). Immunoreactivity was absent in the zona fasciculata (ZF). [C] ATF3 immunoreactivity was diffusely detected in tumor cells of aldosterone-producing adenomas (APA; $n = 20$) and [D] cortisol-producing adenomas (CPA; $n = 5$). Magnification: 100 \times ; size bar = 100 μm .

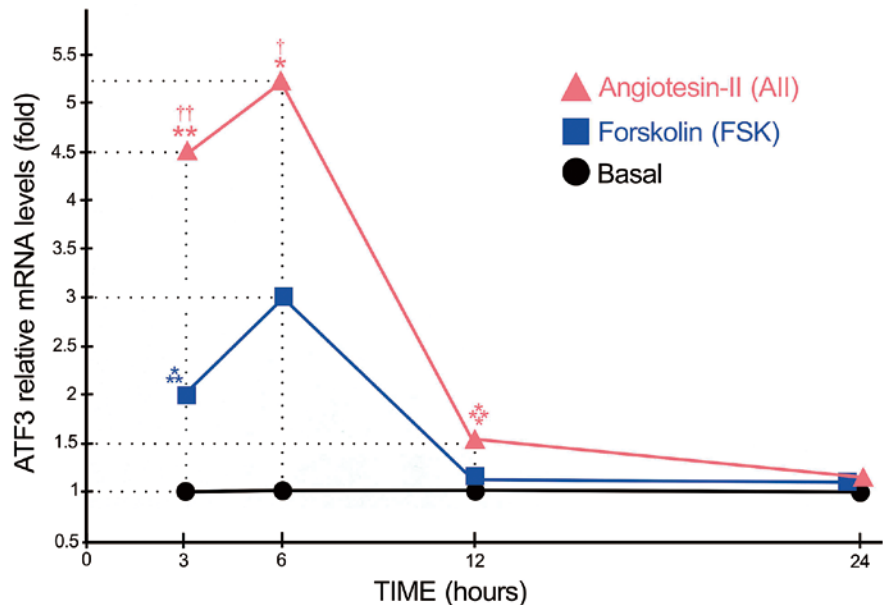


Fig. 3. Expression levels of ATF3 mRNA in time-dependent experiments using H295R cells. Angiotensin-II (AII) or forskolin (FSK) elevated the mRNA levels of ATF3, with peaks at the 6-h time point. Non-treated cells were used as basal controls. *and ** $P < 0.0001$ (AII vs. basal); *** $P = 0.012$ (FSK vs. basal); **** $P = 0.011$ (AII vs. basal); † $P < 0.001$ (AII vs. FSK); †† $P < 0.0001$ (AII vs. FSK).

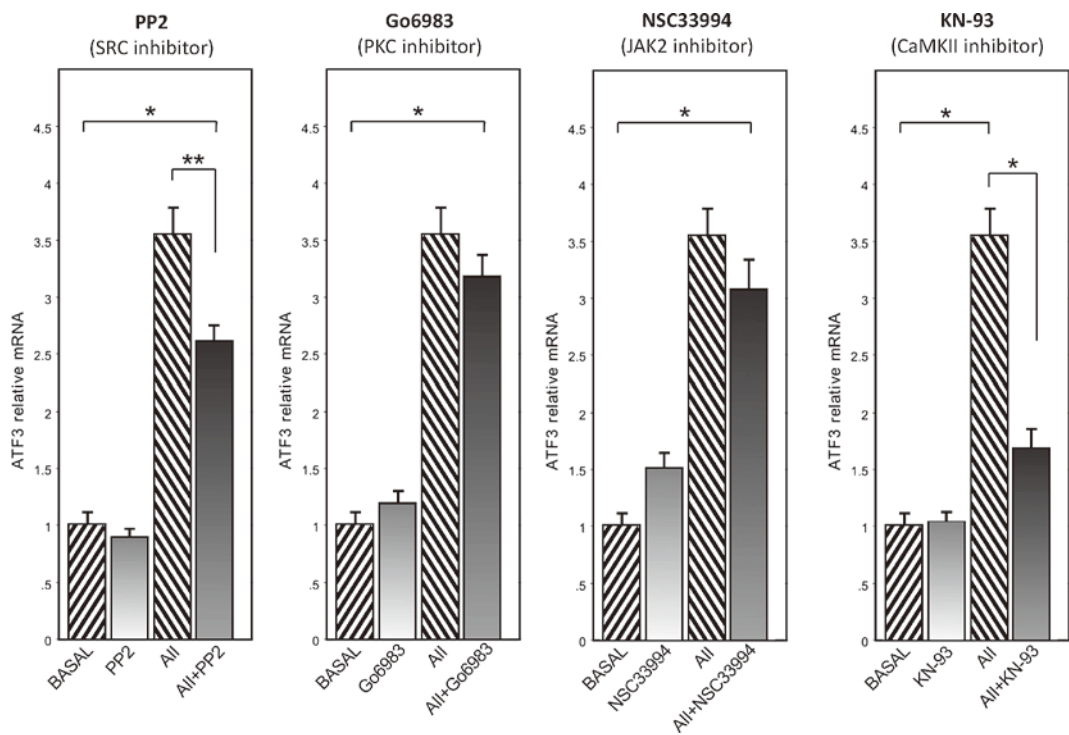


Fig. 4. Inhibition of intracellular pathways in H295R cells. Inhibitors of SRC (PP2), PKC (Go6983), JAK2 (NSC33994) and CaMKII (KN93) were used alone or in combination with angiotensin-II in 6-h treatment experiments. Non-treated groups of cells were used as basal controls. There was a significant decrease in the mRNA levels of ATF3 in cells cotreated with the SRC inhibitor ($P = 0.0002$) or CAMKII inhibitor ($P < 0.0001$) in the presence of angiotensin-II (AII), compared to AII alone. Cells treated with AII showed statistically significant increases in ATF3 levels compared to groups in which AII was absent ($P < 0.0001$), except for the cells treated with KN93 + AII.

can be influenced by the cascade of events triggered by AII in at least two of the above pathways. We also demonstrate that the expression levels of ATF3 mRNA can be induced by FSK, as a response to the stimulus of cAMP during treatment with this agent (Felizola et al. 2014d). This response to FSK can be expected, due to the status of ATF3 as a member of the CREB protein family of transcription factors.

The detection of higher expression levels of ATF3 mRNA in APA compared to CPA in our samples corroborates our results *in vitro* and suggests a possible correlation between this transcription factor and aldosterone biosynthesis in human adrenocortical tissues. In addition, the detection of ATF3 protein in the zona glomerulosa of NA and IHA in our immunohistochemical analysis is consistent with our *in vitro* results and suggests a role for that protein in aldosterone-producing cells of the adrenal cortex. Nevertheless, the immunolocalization of ATF3 in adrenocortical adenomas showed a diffuse distribution in immunoreactivity not only in APA, but also in CPA. Because we were unable to quantify the protein levels in these tumors using immunohistochemistry, we only suggest the higher expression levels of ATF3 in APA based on our qPCR data. The apparently paradoxical expression of ATF3 in the tumor cells of CPA at both mRNA and protein levels can be reasonably explained by the results of previous studies that suggest the involvement of ATF3 in human tumorigenesis (Hunt et al. 2012). That may be a possible reason for the expression of ATF3 in CPA, despite the lack of ATF3 protein in the zona fasciculata of the adrenal cortex (Fig. 2A, B).

At least two roles have been proposed for ATF3 in human cells: as a mediator of stress response and as a regulator of cell proliferation (Hunt et al. 2012). However, it has been postulated that ATF3 can have opposite effects in different cell types (Hunt et al. 2012). Like several other proteins previously reported (Lefebvre et al. 2002; Ye et al. 2007; Felizola et al. 2014a,b,c,d), ATF3 is present in the central nervous system (CNS), in the zona glomerulosa of NA and adrenals with IHA, and in the tumor cells of APA. Previous studies have shown that ATF3 is expressed at relatively low levels in most intact CNS neurons *in vivo*, where it is mainly present in the cytoplasm (Seijffers et al. 2007; Hunt et al. 2012), with a minority of neurons expressing ATF3 in the nuclei (Seijffers et al. 2007). In human adrenocortical tissues, ATF3 protein expression was localized to the cytoplasm, indicating a possible extra-nuclear role for this transcription factor in adrenocortical cells. Other CREB protein family members, like ATF2, can be found in both the nuclei and cytoplasm, and have transcription-independent functions outside of the nucleus, like mitochondrial membrane organization, thereby highlighting the diverse location-dependent functions of this group of proteins (Cho et al. 2001; Bhoumik et al. 2005, 2008; Lau et al. 2012; Lau and Ronai 2012).

However, the detailed functions of ATF3 in the CNS

and other human tissues remain far from clear (Hai and Hartman 2001; Hai et al. 2010; Hunt et al. 2012). The analysis of our results suggests that, in human adrenocortical tissues, ATF3 may play a role in aldosterone synthesis pathways, especially those regulated via CaMKII and SRC, in agreement with previously reported findings (Nogueira et al. 2007, 2009). It is possible that ATF3 can have additional functions in these tissues, but further studies are required for clarification.

Conflict of Interest

The authors have no conflict of interest.

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