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Role of CREB in the regulatory action of sarsasapogenin on muscarinic M_1 receptor density during cell aging

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ABSTRACT

This work studied the role of cyclic AMP responsive element binding protein (CREB) in the up-regulation of M_1 muscarinic acetylcholine receptor (M_1 receptor) density by sarsasapogenin (ZMS) in CHO cells transfected with M_1 receptor gene (CHOm1 cells). During cell aging, sarsasapogenin elevated M_1 receptor density as well as CREB and phosphor-CREB (pCREB) levels. CREB peaked earliest, followed by pCREB and M_1 receptor density peaked last. When CREB synthesis was blocked by antisense oligonucleotides, the elevation effect of sarsasapogenin on M_1 receptor density was abolished. These results suggest that sarsasapogenin up-regulates M_1 receptor density in aged cells by promoting CREB production and phosphorylation. Furthermore, the results support the hypothesis that pCREB regulates M_1 receptor gene expression through heterodimer formation.

1. Introduction

Memory formation requires transcription and translation of new mRNAs, among which those mediated by the transcription factor cyclic AMP responsive element binding protein (CREB) are thought to be prime candidates. Conkright et al. [1] performed an analysis of CREB target genes and identified approximately 70 putative candidates, finding that many of them were important for neuronal specification, axon guidance, synapse formation, and neurotransmitter release [2]. Postnatal disruption of CREB leads to progressive neurodegeneration [3], whereas elevation of CREB protein levels by somatic cell gene transfer attenuates the long-term memory impairment that is associated with normal aging [4]. In order for CREB to activate transcription, CREB must be phosphorylated [5], resulting in the active form phosphor-CREB (pCREB), which then binds to the cyclic AMP responsive element (CRE) of target genes as well as to an adaptor protein (or mediator) known as CREB-binding protein (CBP) that, in turn, contacts and activates the basal transcriptional apparatus [6].

The relationship between CREB phosphorylation and muscarinic acetylcholine receptor (M receptor) expression is far from fully understood. It has been reported [7,8] that the regulatory elements of the muscarinic receptor subtype 1 (M_1 receptor) gene located at the promoter region lacked the CRE sequence (TGACGTCA) but contained the activator protein-1 (AP-1) binding element sequence (TGACTCA). Since CREB and AP-1 can form hetero-dimers to activate the transcription of target genes [9,10], it is reasonable to propose that the phosphorylation of CREB may enhance M_1 receptor expression. However, this hypothesis needs to be proven by experimental results.

Sarsasapogenin (ZMS), is an active ingredient in *Rhizoma anemarrhenae*, a medicinal herb frequently used in traditional Chinese medicine to treat certain chronic diseases including Alzheimer's disease and other memory deficits associated with aging. Its chemical structure is 5β, 20α, 22α, 25S-spirostan-3β-ol. Previous research has demonstrated that ZMS is neither a cholinesterase

Abbreviations: CREB, cyclic AMP responsive element binding protein; CRE, cyclic AMP responsive element; pCREB, phosphor-CREB; CBP, CREB-binding protein; M receptor, muscarinic acetylcholine receptor; M_1 receptor, muscarinic receptor subtype 1; ZMS, sarsasapogenin; DMSO, dimethylsulfoxide; CHOm1 cell line, CHO cells transfected with M_1 receptor gene; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride; AP-1, activator protein-1; BDNF, brain derived neurotrophic factor; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase

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inhibitor nor an M receptor agonist, but could ameliorate the decline of M receptor density in brains and improve the memory of aged rats and certain neurodegeneration models [11]. The purpose of this research was to determine whether CREB plays a role in the regulatory action of ZMS on M_1 receptor density, and furthermore how it might do this; neither of these issues has not been studied previously. We first examined the effect of ZMS on CREB/pCREB expression and M_1 receptor density in CHO cells that were transfected with the M_1 receptor gene. We then compared the time courses of the ratios of CREB, pCREB and M_1 receptor density in ZMS and dimethylsulfoxide (DMSO) treated groups. Finally, we examined whether ZMS could still up-regulate the M_1 receptor density when CREB expression was blocked by specific antisense oligonucleotides.

2. Materials and methods

2.1. Cell culture and treatment

The CHO cells transfected with M_1 receptor gene (CHOm1 cell line) was generously provided by Professor Buckley from University College London, and cells were subsequently cultured following his protocol [12]. In brief, the cells were sub-cultured in Dulbecco's modified Eagle's medium containing proline. The cells stopped growing at 90% confluence and the M receptor density began to decrease due to cell aging. At this point, ZMS (2×10^{-3} M in DMSO, Sigma, 98% purity) was added to a final concentration of 10^{-5} M, and samples were examined for M_1 receptor density and CREB/pCREB content after 12 h, 24 h, 48 h, 72 h and 96 h.

2.2. Western blot of CREB and pCREB

Sodium dodecyl sulfate (SDS)-lysed cells were separated on SDS–polyacrylamide gels. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, blocked in skim milk, and incubated overnight at 4 °C in primary antibody (rabbit anti-CREB antibody, 1:200, Santa Cruz, or mouse anti-pCREB antibody, 1:1000, Upsate, or anti-β-actin antibody, 1:1000, Santa Cruz), which after washing was then followed by incubation in secondary antibody and finally colour-development with ECL reagents (Pierce). Densitometric quantification was performed with an image analyzer (Gel Doc 2000, Bio-Rad). The gray values were ultimately normalised to β-actin.

2.3. Transfection of CHOm1 cells with antisense oligonucleotides of CREB

The antisense oligonucleotide for CREB 5′-TGGTCATCTAGTCACCGGTG-3′ [13] was synthesised by Shanghai Sangon Biotechnology. It matches as the reverse complement of nucleotides 27–46 (GenBank accession no. X14788 [GenBank]). The sense sequence 5′-CACCGGTGACTAGATGACCA-3′ showed no significant match and thus served as a non-functional control. Transfection was carried out using the Fugene 6 Transfection Reagent kit (Roche) following instructions in the kit protocol. In brief, when the cells were 70% confluent, 1μg of oligonucleotides and 3 μl transfection reagent were added to 10^5 cells in 1 ml culture medium and were then incubated for 4 h to complete the transfection. CREB, pCREB and M_1 receptor density were examined 48 h later.

2.4. ^{3}H-QNB binding assay for M receptor density

M receptor density was measured using the ^{3}H-QNB single point method, as modified by Hu et al. [11]. Non-specific binding was measured with a parallel assay containing atropine. Given that the M receptor in CHOm1 cells were exclusively subtype 1, the specific binding of ^{3}H-QNB represented the M_1 receptor density.

2.5. Statistical analysis

The data are presented as mean ± S.E.M. Statistical analysis was carried out using the SAS software package. Two-way ANOVA with post hoc Newman–Keuls test was used for experiments involving treatment and time of more than two groups. Paired Student's *t*-tests were used for comparisons between two experimental groups. $P < 0.05$ was considered as significant.

3. Results

3.1. ZMS increases CREB and pCREB expression in CHOm1 cells

As shown in Fig. 1A, Western blotting revealed that CREB and pCREB were both significantly elevated by ZMS. The differences between ZMS treatment and DMSO solvent control were highly

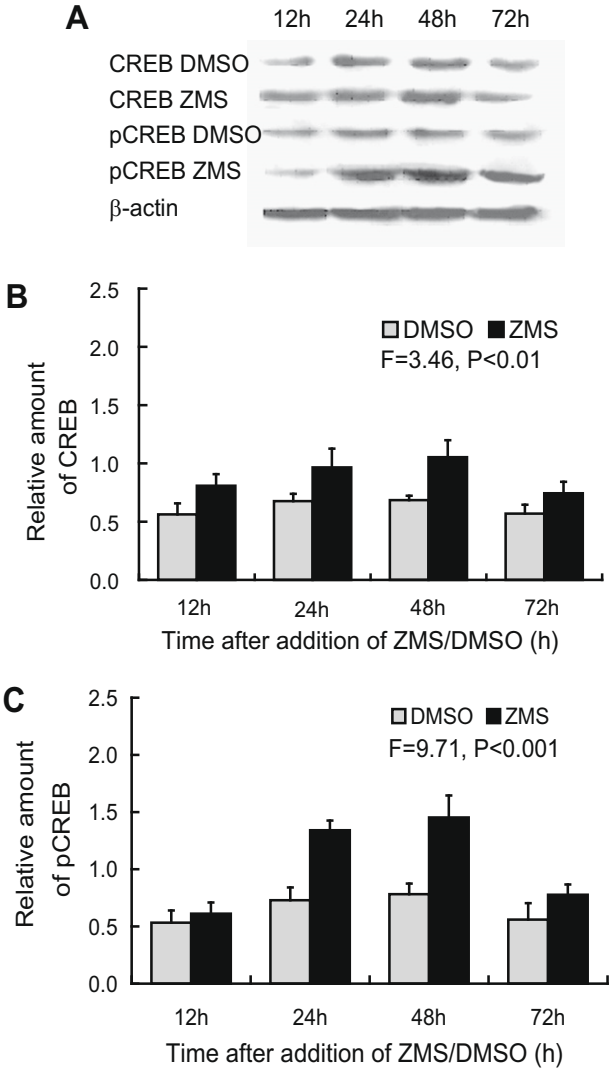

Fig. 1. Effect of ZMS on CREB and pCREB expression in CHOm1 cells. (A) Western blot showing CREB and pCREB in extracts of cells treated with ZMS/DMSO or DMSO alone. (B and C) Results of two-way ANOVA for CREB and pCREB expression in ZMS/DMSO or DMSO control-treated groups. Data were expressed as the mean ± S.E.M (the respective bands of the DMSO-treated group at 0 h were set as 0). The *F* value and *P* value between treatments were indicated in the figure.