

SUMO1 enhances 17- β estradiol's effect on CRH promoter activation through estrogen receptors

Hong ZHU, Jiang-Ning ZHOU*

1. Hefei National Laboratory for Physical Sciences at Microscale, and Department of Neurobiology, School of Life Science, University of Science and Technology of China.

Correspondence to: Jiang-Ning Zhou, MD PhD
Hefei Jinzhai Road 96, Anhui 230026, P.R. China
TEL: +86 551 3607658; FAX: +86 551 3600408
E-MAIL: jnzhou@ustc.edu.cn

Submitted: 2007-12-02 Accepted: 2008-03-05 Published online: 2008-04-18

Key words: corticotrophin-releasing hormone, CRH; 17- β estradiol, E₂; estrogen receptor, ER; small ubiquitin related modifier-1, SUMO-1; hypothalamic-pituitary-adrenal axis, HPA axis

Neuroendocrinol Lett 2008; 29(2):230-234 PMID: 18404132 NEL290208A08 ©2008 Neuroendocrinology Letters • www.nel.edu

Abstract

OBJECTIVES: Human corticotrophin-releasing hormone (CRH) plays a pivotal role in the stress response. Its expression is under the control of various steroid hormones, such as estrogens. The transcriptional activity of the estrogen receptor (ER) may be modified by small-ubiquitin related modifier (SUMO1). In the present study, we aim to reveal the role of SUMO1 in the regulation of ER-mediated CRH promoter activation.

METHODS: CHO-K1 cells were transfected with human ER and SUMO1 expressing plasmids together with the CRH promoter reporter gene. CRH mRNA was detected in BE(2)C cells by real time PCR.

RESULTS: We found that estradiol could elevate CRH promoter activity to a much higher level in cells co-transfected with ER and SUMO1 than that with ER alone, and that the enhancement was blocked by the ER inhibitor, ICI182,780. Furthermore, the endogenous CRH mRNA expression was also increased when the BE(2)C cell were transfected with ER α and SUMO1 in contrast to the transfection with ER α alone.

CONCLUSIONS: Our results indicate that SUMO1 participates in the modulation of ER-mediated CRH mRNA expression which may be important for the regulation of the stress response.

Abbreviations

SUMO - small ubiquitin related modifier
ER - estrogen receptor
ERE(s) - estrogen responsive element(s)
CRH - corticotrophin-releasing hormone
HPA - hypothalamic-pituitary-adrenal

Corticotrophin-releasing hormone (CRH), a 41-amino acid peptide, plays a pivotal role in the regulation of the stress response. In the central nervous system (CNS), CRH can regulate the expression of proopiomelanocortin (POMC)[1], adrenocor-

trophic hormone (ACTH)[2] and the release of some other factors [3; 4]. Thus, it regulates the activity of hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic autonomous system[5]. Stress sends signals to CRH-expressing cells and thereby stimulates CRH expression and release. These stress signals contain a variety of biochemical agents, including steroid hormones, such as estrogens. Estrogens have been implicated in the regulation of CRH synthesis. *Vamvakopoulos et al* have found that ER can bind to the perfect

half-palindromic estrogen responsive elements (EREs) sequence within the CRH promoter and also reported 17- β estradiol (E_2) could activate the CRH promoter through ER.[6] Estrogens have also been reported to modulate CRH expression in the hypothalamic paraventricular nucleus of ovariectomized rats.[7] Bao *et al* have shown the co-localization of ER α and CRH in the human hypothalamic paraventricular nucleus (PVN) in mood disorder. [8] Increasing evidence shows that the estrogen receptor may be a key regulator of CRH and the HPA axis. However, how the modification of ER itself at the transcription or post-translation level may affect on the CRH expression is not yet clear.

An increasing number of studies suggest that post-translational modifications play an important additional part in the functions of steroid receptors.[9] SUMOylation is the recently discovered to cause post-translational modifications, during which a small protein named small ubiquitin related modifier (SUMO) is conjugated to a substrate by several enzymes. The interaction between ER and SUMO1 has been demonstrated recently. *Chauchereau* and his college showed estrogen receptor α (ER α)-driven transcription was enhanced by SUMO-1 over-expression.[10] Recently, ER α was reported to interact with UBC9 and PIAS1, a SUMO1 conjugating enzyme and ligase, respectively. In their study, *Sentis et al* have detected a direct interaction of ER α and SUMO1 using pull down and co-immunoprecipitate assays.[11]

Many studies shows that SUMOylation of nuclear receptors occurs during stress such as hypoxia, heat shock, oxidative stress, ethanol and many other stress conditions.[12] On the other hand, SUMO1, a member of SUMO family, in the brain or cell lines increased during hypoxia which indicates that it could be involved in the stress response and then may be involved in the regulation of HPA-axis. [13; 14]

In the present study, we aimed to demonstrate that SUMO1 is involved in CRH promoter activation. We found that over-expression of SUMO1 enhances ER driven CRH activation with their ligand 17- β estradiol, although there are no complete estrogen responsive element (EREs). In the CRH promoter. These novel findings warrant further investigations into the post-translational modification of steroid receptors in the regulation of the HPA axis and the molecular mechanisms of the stress response.

MATERIALS AND METHODS

Plasmid constructions

The human SUMO1 cDNA fragment was PCR amplified from pcDNA4-SUMO1 kindly donated by Prof. Wang Guang-Hui from University of Science and Technology of China (USTC, PRC). The inserts were cloned between XhoI and BamHI into a pEGFP-C1 vector. The CRH promoter was PCR amplified from human

blood DNA and cloned between BglII and EcoRI into a pGL luciferase vector. The primers from region -3652 to +124bp were synthesized according to the human CRH 5' flanking sequence (GeneBank X67661) and the human CRH gene sequence (GeneBank AF48855). The vector expressing human ER (pSG-ER α) were donated by Jan-Ake Gustafsson (Karolinska Institute, Sweden).

Cell culture and transfection

CHO-K1 cells were maintained in phenol red-free DMEM/F-12 (Sigma) and 10% heat-inactivated charcoal-stripped New Born Culf Serum (NBS) (Gibco) in 5% CO₂ at 37 °C. Before transfection, the cells were sub-cloned in 24-well plates (Corning). After washing with D-PBS (Gibco), cells were at 80% confluence transfected with LipofectAmine 2000 (Invitrogen) according to manufacturer's instructions. The total amount of DNA in each well was 0.25 μ g, including 100ng pSG-ER α , 50ng CRH-LUC reporter and 100ng pEGFP-SUMO1 together with 0.25ng pRL-TK Renilla luciferase vector as internal control. Each well was made up to the same plasmid amount with empty vectors. Cells were then treated with 17- β estradiol (Sigma), ICI 182 780 (Tocris) or vehicle (ethanol) for 24 hours.

BE(2)C cells purchased from ATCC were maintained in DMEM/F-12 (Sigma) and 10% fetal bovine serum (PAA) in 5% CO₂ at 37 °C. Transfections were performed as described above. BE(2)C cells were transfected with 500ng pEGFP or 500ng pEGFP-SUMO1 together with pSG5-ER α . The cells were refreshed with completed medium 6 hours later after transfection.

Cell harvest and luciferase assay

24hours after treatment, the cells were washed twice with PBS and lysed with 100ul Passive Lysis Buffer (Promega) and a 20ul aliquot was used for the luciferase assay with the Dual-luciferase activity assay kit (Promega). Luciferase activities were normalized to the internal control Renilla luciferase activity.

RNA isolation and real-time PCR

Total RNA was isolated from cultured BE(2)C cells (ATCC) using Trizol Reagent (Invitrogen) according to the manufacture's instructions. All the extracted RNA samples were digested by RNase-free DNase (promega).

RT and PCR procedures were carried out separately. Equal amounts of total RNA (2 μ g/each) were mixed with 1 μ l oligo(dt) and heated at 72 °C for 5min. Then 1 μ l dNTP (10mM) and 1U RNase Inhibitor and 1 \times MMLV buffer were added and incubated at 37 °C for another 5min. After being chilled on ice, the mix was added with 1U M-MLV and incubated at 42 °C for 60min. The reaction was terminated at -20 °C for 10min and stored at -80 °C.

For real time PCR analysis, Amplification reactions were performed in duplicate, with the ABI PRISM 7000 Real Time polymerase chain reaction (PCR) Sys-

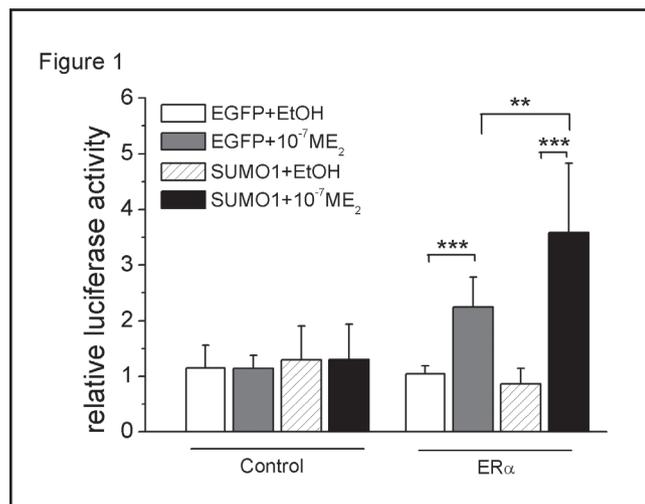


Fig. 1. Effect of SUMO1 over-expression on ER-induced CRH promoter activation. CHO cells were co-transfected with 50ng CRH-Luc, 100ng pEGFP-SUMO1 or pEGFP-C1 empty vector together with 100ng pSG-ER α . 0.25ng pRL-TK Renilla luciferase vector was used as internal control. After transfection, cells were treated with 10^{-7} M E $_2$ for 24 hours. Mean \pm S.E.M. from at least three independent experiments are shown. *** $p < 0.001$ ** $p < 0.01$

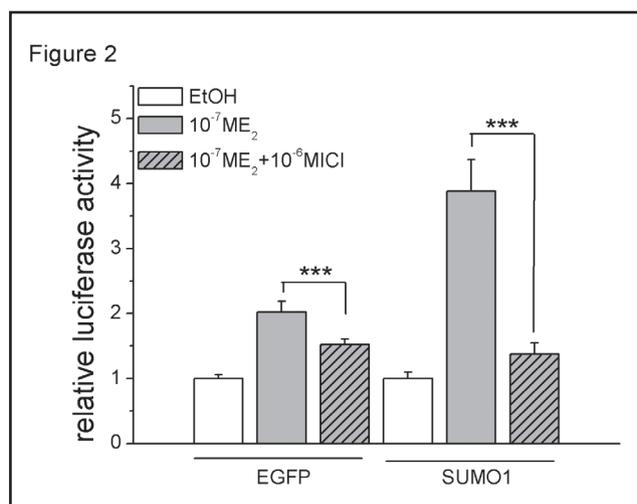


Fig. 2. ICI 182,780 inhibits E $_2$ effects with or without SUMO1. CHO cells were treated with 10^{-7} M E $_2$ and 10^{-6} M ICI 182 780, an ER selective antagonist. Mean \pm S.E.M. from at least three independent experiments are shown. *** $p < 0.001$

tem (Applied Biosystems, Foster City, California). 1 μ l cDNA sample was amplified in 50 μ l 1 \times Power SYBR Green PCR Master Mix (ABI) and 1nM of each primer for human CRH gene (5'-CATCTCCCTGGATCTCACCTTC-3' (forward) and 5'-ATAATCTCCATGAGTTTCTGTTG-3' (reversed)) or human β -actin gene (5'-TTGCCGACAGGATGCAGAA-3' (forward) and 5'-GCCGATCCACACGGAGTACTT-3' (reversed))

The cycling protocol was as followed: 50 $^{\circ}$ C 2min, 95 $^{\circ}$ C 10min, followed by 40 cycles of 95 $^{\circ}$ C 15sec, 60 $^{\circ}$ C 1min.

Statistical analysis

The data are presented as Means \pm SEM from at least three independent experiments in duplicated wells. The significant difference between the means was calculated by Two-Way ANOVA followed by Independent sample T-test.

RESULTS

ER mediated CRH promoter activation could be enhanced by SUMO1

We have found E $_2$ -induced upregulation of CRH promoter through ER α . As shown in Figure 1, in the absence of ER there is no difference of CRH promoter activity between 17 β -estradiol (E $_2$) and ethanol (EtOH) control with or without SUMO1 over-expression. In the presence of ER α E $_2$ could only enhance CRH promoter activity for around two times. Interestingly, when SUMO1 was co-transfected into the cells, E $_2$

enhanced CRH promoter activity up to around 5-fold through ER α . Their transcriptional activities in CRH promoter regulation were greatly increased by SUMO1 in the presence of ligand. Two-Way ANOVA revealed a significant effect of E $_2$ [F(1,23)=47.718; $p < 0.001$] and a trend in significance of SUMO1 [F(1,23)=2.019; $p = 0.054$]. The interaction of E $_2$ by SUMO1 was also significant [F(1,23)=7.196; $p < 0.05$].

ER antagonist inhibits SUMO1 enhanced CRH transcriptional activity

To further confirm ER's involvement we used ER antagonist ICI 182,780 to inhibit ER function during the experiment. To determine if SUMO1 enhanced CRH activation could be inhibited by ER antagonist, cells were treated with 10^{-7} M E $_2$ or 10^{-7} M E $_2$ together with 10^{-6} M ICI 182 780. EtOH was used as a control vehicle. As shown in Figure 2 ICI 182 780 significantly decreased E $_2$ evoked CRH promoter activation either in the absence or in the presence of SUMO1 ($p < 0.001$).

SUMO1 elevated endogenous CRH mRNA expression in BE(2)C cell line

To further examine the role of SUMO1 on the endogenous CRH mRNA expression, we analyzed CRH mRNA expression in BE(2)C cells by real-time RT-PCR. As shown in figure 3, CRH mRNA expression in cells transfected with ER α and SUMO1 was significantly higher in contrast to those transfected with ER α alone. ($p < 0.05$)

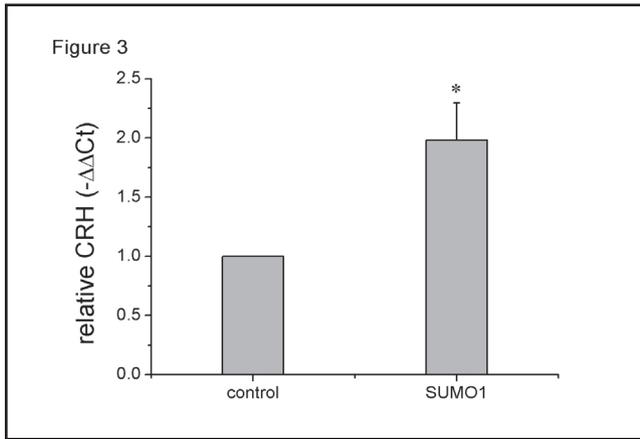


Fig.3 SUMO1 increases endogenous CRH expression in BE(2)C cells. BE(2)C cells were transfected with 500ng pSG5-ER α and 500ng pEGFP-SUMO1, empty vector was used as control. Mean \pm S.E.M. from at least three independent experiments are shown. * $p < 0.05$

DISCUSSION

Taken together, our findings shows that SUMO1 affects ER α 's modulatory functions on CRH promoter in a ligand dependent way, which indicates that post-translational modification of ER α is involved in HPA-axis regulation and may even participate in the stress response and mood disorder. Evidence is accumulating on the essential role of SUMO and SUMOylation in brain functions. Immunohistochemistry and in situ hybridization have revealed the presence of SUMO and SUMO related proteins in the human supraoptic nucleus of the hypothalamus (SON) [16], which expresses vasopressin and other neuropeptides, in physiological circumstances. We also found a high level of expression of SUMO1 in several nuclei of the rat hypothalamus (data not shown). Increasing evidence points also to a role of SUMO1 and SUMOylation. SUMO1 in pathological conditions since it is increased during the exposure to many stressors, such as hypoxia [13; 14], ischemia [17], heat shock, ethanol, the amino acid analog canavanine[18; 19], electropile, oxidative stress[12] and so on. Increased expression of SUMO1 and elevated SUMOylation levels of proteins involved in neurodegenerative diseases are demonstrated during these circumstances [20; 21]. Kane and his college showed after exposure to nicotine for 14 days, SUMO1 was upregulated in rat prefrontal cortex (PFC) while it was downregulated in the medial basal hypothalamus (MBH).[22] Interestingly, the PFC and the hypothalamus are both involved in HPA-axis regulation and stress responses. These are also two critical parts of brain in the pathogenesis of depression [23]. It has been reported that CRH receptors and GABA receptors mRNA were up-regulated in patients who committed suicide while the CRH levels were elevated in the prefrontal cortex[24]. It has also been demonstrated that there is increased CRH production by the neurons in the hypothalamic paraventricular nucleus (PVN) of depressed patients[25]. And previous study in our group discovered that the increased number of CRH positive neurons in PVN of patients with mood disor-

ders is combined with an increased ER α colocalization in CRH neurons.[8] Our present findings suggest that SUMO1 may be involved in ER-mediated CRH synthesis and in the regulation of the activity of HPA axis.

ACKNOWLEDGEMENTS

We would like to thank Dr. J.A. Gustafsson for his kindness in providing the pSG5-ER α plasmids and Dr. M. Wu for gifting the pRL-CMV plasmid. This research was supported by the Ministry of Science and Technology of China (2006CB500705) and Natural Science Foundation of China (30530310).

REFERENCES

- Boutillier AL, Gaiddon C, Lorang D, Roberts JL, and Loeffler JP (1998). Transcriptional activation of the proopiomelanocortin gene by cyclic AMP-responsive element binding protein. *Pituitary* **1**: 33-43.
- Aguilera G, Nikodemova M, Wynn PC, and Catt KJ (2004). Corticotropin releasing hormone receptors: two decades later. *Peptides* **25**: 319-29.
- Groef BD, Goris N, Arckens L, Kuhn ER, and Darras VM (2003). Corticotropin-releasing hormone (CRH)-induced thyrotropin release is directly mediated through CRH receptor type 2 on thyrotropes. *Endocrinology* **144**: 5537-44.
- Groef BD, Van der Geyten S, Darras VM, and Kuhn ER (2006). Role of corticotropin-releasing hormone as a thyrotropin-releasing factor in non-mammalian vertebrates. *Gen Comp Endocrinol* **146**: 62-8.
- Minton JE (1994). Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. *J Anim Sci* **72**: 1891-8.
- Vamvakopoulos NC, and Chrousos GP (1993). Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression. Potential implications for the sexual dimorphism of the stress response and immune/inflammatory reaction. *J Clin Invest* **92**: 1896-902.
- Paulmyer-Lacroix O, Hery M, Pugeat M, and Grino M (1996). The modulatory role of estrogens on corticotropin-releasing factor gene expression in the hypothalamic paraventricular nucleus of ovariectomized rats: role of the adrenal gland. *J Neuroendocrinol* **8**: 515-9.
- Bao AM, Hestiantoro A, Van Someren EJ, Swaab DF, and Zhou JN (2005). Colocalization of corticotropin-releasing hormone and oestrogen receptor- α in the paraventricular nucleus of the hypothalamus in mood disorders. *Brain* **128**: 1301-13.

- 9 Faus H, and Haendler B (2006). Post-translational modifications of steroid receptors. *Biomed Pharmacother* **60**: 520–8.
- 10 Chauchereau, Amazit L, Quesne M, Guiochon-Mantel A, and Millgrom E (2003). Sumoylation of the progesterone receptor and of the steroid receptor coactivator SRC-1. *J Biol Chem* **278**:12335–43.
- 11 Sentis S, Le Romancer M, Bianchin C, Rostan MC, and Corbo L (2005). Sumoylation of the estrogen receptor α hinge region regulates its transcriptional activity. *Mol Endocrinol* **19**: 2671–84.
- 12 Manza LL, Codreanu SG, Stamer SL, Smith DL, Wells KS, Roberts RL, and Liebler DC (2004). Global shifts in protein sumoylation in response to electrophile and oxidative stress. *Chem Res Toxicol* **17**: 1706–15.
- 13 Shao RJ, Zhang FP, Tian F, Friberg PA, Wang XY, Sjolund H, and Billig H (2004). Increase of SUMO-1 expression in response to hypoxia: direct interaction with HIF-1 α in adult mouse brain and heart in vivo. *Febs Letters* **569**: 293–300.
- 14 Comerford KM, Leonard MO, Karhausen J, Carey R, Colgan SP, and Taylor CT (2003). Small ubiquitin-related modifier-1 modification mediates resolution of CREB-dependent responses to hypoxia. *PNAS* **100**: 986–991.
- 15 Kobayashi S, Shibata H, Yokota K, Suda N, Murai A, Kurihara I, Saito I, and Saruta T (2004). FHL2, UBC9, and PIAS1 are novel estrogen receptor α -interacting proteins. *Endocr Res* **30**: 617–21.
- 16 Villagra NT, Navascues J, Casafont I, Val-Bernal JF, Lafarga M, and Berciano MT (2006). The PML-nuclear inclusion of human supra-optic neurons: a new compartment with SUMO-1- and ubiquitin-proteasome-associated domains. *Neurobiol Dis* **21**: 181–93.
- 17 Lee YJ, Miyake S, Wakita H, McMullen DC, Azuma Y, Auh S, and Hallenbeck JM (2007). Protein SUMOylation is massively increased in hibernation torpor and is critical for the cytoprotection provided by ischemic preconditioning and hypothermia in SHSY5Y cells. *J Cereb Blood Flow Metab* **27**: 950–62.
- 18 Kurepa J, Walker JM, Smalle J, Gosink MM, Davis SJ, Durham TL, Sung DY, and Vierstra RD (2003). The small ubiquitin-like modifier (SUMO) protein modification system in Arabidopsis - Accumulation of SUMO1 and -2 conjugates is increased by stress. *Journal of Biological Chemistry* **278**: 6862–6872.
- 19 Hietakangas V, Ahlskog JK, Jakobsson AM, Hellesuo M, Sahlberg NM, Holmberg CI, Mikhailov A, Palvimo JJ, Pirkkala L, and Sistonen L (2003). Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. *Mol Cell Biol* **23**: 2953–68.
- 20 Ueda H, Goto J, Hashida H, Lin X, Oyanagi K, Kawano H, Zoghbi HY, Kanazawa I, and Okazawa H (2002). Enhanced SUMOylation in polyglutamine diseases. *Biochem Biophys Res Commun* **293**: 307–13.
- 21 Steffan JS, Agrawal N, Pallos J, Rockabrand E, Trotman LC, Slepko N, Illes K, Lukacsovich T, Zhu YZ, Cattaneo E, Pandolfi PP, Thompson LM, and Marsh JL (2004). SUMO modification of Huntingtin and Huntington's disease pathology. *Science* **304**:100–4.
- 22 Kane JK, Konu O, Ma JZ, and Li MD (2004). Nicotine coregulates multiple pathways involved in protein modification/degradation in rat brain. *Brain Res Mol Brain Res* **132**: 181–91.
- 23 Swaab DF, Fliers E, Hoogendijk WJ, Veltman DJ, and Zhou JN (2000). Interaction of prefrontal cortical and hypothalamic systems in the pathogenesis of depression. *Prog Brain Res* **126**: 369–96.
- 24 Merali Z, Du L, Hrdina P, Palkovits M, Faludi G, Poulter MO, and Anisman H (2004). Dysregulation in the suicide brain: mRNA expression of corticotropin-releasing hormone receptors and GABA(A) receptor subunits in frontal cortical brain region. *J Neurosci* **24**: 1478–85.
- 25 Raadsheer FC, Hoogendijk WJ, Stam FC, Tilders FJ, and Swaab DF (1994). Increased numbers of corticotropin-releasing hormone expressing neurons in the hypothalamic paraventricular nucleus of depressed patients. *Neuroendocrinology* **60**: 436–44.