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ERp44 Mediates Gene Transcription *via* Inositol 1, 4, 5-Trisphosphate Receptors in HeLa Cells*

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Abstract Increase in nuclear calcium concentration has several biological effects which include controlling calcium-activated gene transcription. Using extracellular ATP to induce intracellular calcium transient as a model, Western blotting, immunofluorescence, real-time PCR as well as Ca^{2+} image studies were carried out. It was found that inositol 1, 4, 5-trisphosphate receptors (IP_3Rs) and endoplasmic reticulum protein 44 (ERp44) co-localized on the nuclear envelope and endoplasmic reticulum (ER). Extracellular ATP induced nuclear calcium transient *via* IP_3Rs and subsequently increased the cAMP response element binding protein (CREB) phosphorylation and the expression of c-Myc. However, all these were inhibited by 2-aminoethoxydiphenyl borate (2-APB), an IP_3Rs inhibitor, and by over-expression of ERp44. These results suggest that ERp44 inhibits gene transcription *via* IP_3Rs in HeLa cells.

Key words ERp44, ATP, inositol 1, 4, 5-trisphosphate (IP_3) receptors, calcium transient, gene transcription
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Extracellular ATP induces an intracellular Ca^{2+} transient by activating phospholipase C (PLC)-associated P2Y purinergic receptors and producing inositol 1, 4, 5-trisphosphate (IP_3) which leads to Ca^{2+} release from intracellular stores [1–3]. P2Y receptors belong to the large group of G-protein-coupled receptors including at least five distinct subtypes of P2Y receptors, which play an important role in Ca^{2+} signaling in response to extracellular ATP[2, 4–5].

Calcium signals have been associated with several functional activities of animals and tissues, for example, the survival of fish [6], the contraction of cardiac and skeletal muscle[7–9], fertilization of eggs, the development of the tadpole [10], and neuromuscular function [11–12]. ERp44 is firstly characterized as an endoplasmic reticulum(ER) resident protein functioning in protein folding, thiol-mediated retention [13–14], and identified as the first regulatory protein of IP_3Rs from the ER lumen side. It has been reported that ERp44 affects calcium transient by binding to and inhibiting IP_3 receptor subtype 1(IP_3R_1)[15].

Changes in nucleoplasmic Ca^{2+} concentration activate nuclear transcription factors in many types of cells[2, 16–19]. There are two major types of intracellular calcium release channels or receptors, rynaodine receptors (RyRs) and IP_3Rs, located on sarcoplasmic reticulum (SR)/ER and nuclear membrane. These two types of Ca^{2+} release channels are responsible for the mobilization and signal transduction of intracellular calcium. Each type of these calcium release channels is further divided into 3 subtypes[20]. Some transcriptional regulators activation is elicited either by RyRs- or by IP_3Rs-mediated Ca^{2+} signals[21]. It has been reported that only IP_3Rs are expressed in HeLa cells[15, 22]. Although the ER and nuclear envelope (ER-NE) have been demonstrated to be as an interconnected Ca^{2+} store[23], the regulation of ERp44 located on ER-NE and the

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role of extracellular ATP-induced nuclear Ca^{2+} transient in gene expression are poorly understood.

In the present study, using confocal microscopy, immunofluorescence, Western blotting, and real-time PCR, we have investigated, for the first time, the role and regulation of ERp44 on gene transcription in HeLa cells. The results demonstrate that ERp44 and extracellular ATP activate gene transcription *via* IP_3Rs, ERp44 inhibits nuclear calcium transient, and consequently down regulates gene expression in HeLa cells.

1 Materials and methods

1.1 Chemicals and reagents

High Dulbecco′s modified Eagle′s medium (H-DMEM), Trizol reagent and Lipofectamine 2000 were purchased from Invitrogen. Fluo-4 AM was from Molecular Probes, and 2-APB was from Calbiochem. Antibodies were obtained from Cell Signaling or Milipore. Fetal bovine serum was obtained from GIBICO, 4, 6-diamidino-2-phenylindole (DAPI) and other chemicals were purchased from Sigma. The SYBR green kit was purchased from Takara.

1.2 Cell culture and cell transfection

HeLa cells were cultured in H-DMEM medium supplemented with 10% fetal bovine serum, 100 mg/L penicillin, and 100 mg/L streptomycin. The full-length of the ERp44′s cDNA was kindly provided by Professor C C Wang. The expression vectors were constructed using the New England Biolabs′s restriction enzymes and the details will be supplied on request. The cells for calcium images, Western blot and real-time PCR were transfected with pCMV-IRES-RED (control) or pCMV-ERp44-IRES-RED (ERp44). The cells for immunofluorescence study were transfected with pDsRed-RDEL-ERp44 by Lipofectamine 2000. Primers for GADPH: 5′ CAGCAAGGACACTGAGC-AAG 3′ and 5′ GGGTGCAGCGAACTTTATTG 3′; IP_3R_1: 5′ AACAATCCACCCAAGAAA 3′ and 5′ A-AGAGCCTCACCACATCA 3′; IP_3R_2: 5′ CCAACC-CACCCAAGAAGT 3′ and 5′ CAGAACGGATGGA-T GTAGAA 3′; IP_3R_3: 5′ CTCATCTCGCTACTC-TTCTGG 3′ and 5′ TGTGCTATCGGGCTCCTC 3′; RyR_1: 5′ GAAGGTTCTGGACAAACACGGG 3′ and 5′ TCGCTCTTGTTGTAGAATTTGCGG 3′; RyR_2: 5′ GACGGCAGAAGCCACTCACCTGCG 3′ and 5′ CCTGCAGAGAAACTGACAACTGG 3′; RyR_3: 5′ AGAAGAGGCCAAAGCAGAGG 3′ and 5′ GGA-GGCCAACGGTCAGA 3′.

1.3 Ca^{2+} image

After being transfected for 48 h, cells were incubated with 10 μm Fluo-4 AM at room temperature for 10 min, and then placed onto the stage of an inverted microscope (SP5, Leica, Germany), using a Plan Apo ×40 oil objective. Cells were perfused in a standard solution containing (in mmol/L) 140 NaCl, 5.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 3 glucose; pH 7.4, adjusted with NaOH. 488 nm and 543 nm were the excitation wave lengths for detection of Ca^{2+} and dsRED, respectively. The sequential scanning model was used in case of the crosstalk of the excitations. In order to inhibit IP_3Rs, cells were incubated with 100 μmol/L 2-APB in the standard extracellular solution for 20 min at room temperature before each experiment. To identify nucleuses and measure the Ca^{2+} transient of nucleuses, cells were treated with 25 mg/L DAPI for 1 h before carrying out the image of Ca^{2+}.

1.4 Immunofluorescence

HeLa cells were fixed with 0.1% paraformaldehyde (wt/vol) and permeabilized with 0.1% Triton X-100 for 10 min, and then incubated with antibodies against IP_3Rs (1 ∶ 100) at room temperature for 2 h. After washing, the cells were incubated with FITC-conjugated anti-rabbit IgG.

1.5 Real-time PCR

Real-time PCR studies were performed using SYBR green kit on Corbett 6200. 20 μl PCR reactions included 1 μl RT product, 0.4 μl primer1, 0.4 μl primer2, 10 μl 1×SYBR green mix and 8.2 μl ddH_2O. The mixtures were incubated for 1 min at 95℃, followed by 35 cycle for 30 s at 95℃, and for 30 s at 55℃. All reactions were run in triplicates. We defined the threshold cycle (CT) as the fractional cycle number at which the fluorescence passed the fixed threshold. CT values were converted to experimental data using a special equation. The primers for c-Myc: 5′ GGAAG-GACTATCCTGCTGCCAA 3′ and 5′ GCTCCAAG-ACGTTGTGTGTTCG 3′; Primers for GAPDH: 5′ ATG GGG AAG GTG AAG GTC G 3′ and 5′ GGG GTC ATT GAT GGC AAC AAT A 3′.

1.6 Western blotting

Total protein extracts were prepared in radioimmunoprecipitation(RIPA) lysis buffer and were transferred onto PVDF membranes after separation by 10% SDS-PAGE. The membranes were blocked with 5% milk in TBST for 1 h, incubated for 2 h with primary antibodies, and then probed for 1 h with

HRP-conjugated anti-rabbit IgG. After extensively washed with TBST, the target proteins were detected on the membranes by enhanced chemiluminescence.

1.7 Data analysis

SIGMAPLOT and ImageJ were used in statistical and imaging analysis. The groups were compared by one-way, repeated measures of ANOVA and significant differences between groups were determined by the Student′s t test for pairwise comparisons. Results are expressed as $\bar{x} \pm s$ where applicable. Only $P < 0.05$ is considered significant.

2 Results

2.1 Expression and distribution of IP_3Rs and ERp44 in HeLa cells

First, by using RT-PCR, we investigated the expression and distribution of IP_3Rs and ERp44 in HeLa cells. The results indicated that only IP_3Rs were present in HeLa cells, as demonstrated in Figure 1a, which is consistent with other groups′ findings [15, 22]. Since ERp44 is an endoplasmic reticulum (ER) luminal protein [13–15], the ER retention signal (RDEL) was used in the present study to construct a plasmid pDsRed-RDEL-ERp44. Our results suggested that a close co-localization of expression and distribution of ERp44 and IP_3Rs on the nuclear envelope and ER existed (Figure 1b), confirming the result of previous studies[1].

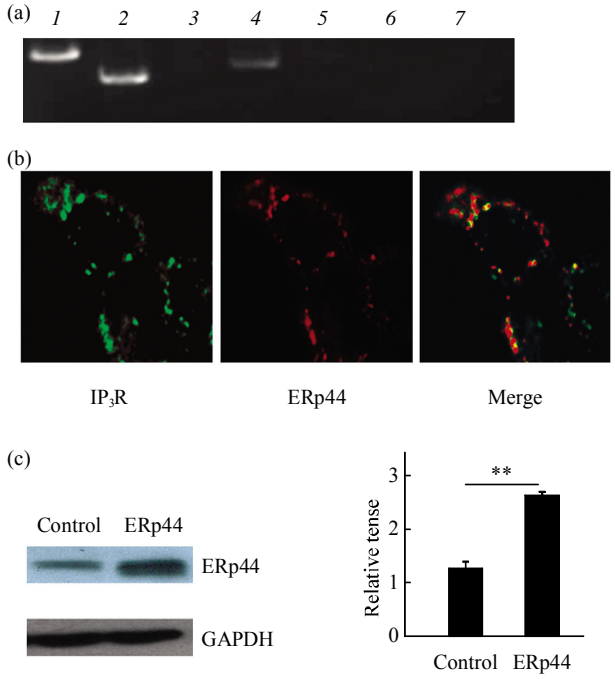

Fig. 1 Expression and distribution of IP_3Rs and ERp44 in HeLa cells

(a) RT-PCR products of HeLa cells, only GAPDH, IP_3R_1, and IP_3R_3 were present in the cells. *1*: GAPDH; *2*: IP_3R_1; *3*: IP_3R_2; *4*: IP_3R_3; *5*: RyR_1; *6*: RyR_2; *7*: RyR_3. (b) Immunofluorescence study was conducted in pDsRed-RDEL-ERp44 transfected HeLa cells. The results showed that ERp44 and IP_3Rs were co-localized on the nuclear envelope and ER lumen. (c) Western blotting studies, n =4, $^{**}P < 0.001$, HeLa cells transfected with pCMV-ERp44-IRES-RED or pCMV-IRES-RED were used and total proteins were collected.

Moreover, the expression and over-expression of ERp44 in HeLa cells were also examined using Western blotting in the present study. The results indicated that ERp44 was successfully over-expressed in HeLa cells in our experiments (Figure 1c), which established the bases for the present study.

2.2 ERp44 inhibits extracellular ATP-induced nuclear calcium transient mediated by IP_3Rs

It has been reported that ERp44 inhibits intracellular Ca^{2+} transient *via* IP_3R_1 in HeLa cells [15], here we first sought to examine this in our experimental system to see if it did the same as reported, therefore. Indeed, application of ATP 10 μmol/L induced a significant intracellular Ca^{2+} transient (data not shown). In the next series of experiments we examined the role of extracellular ATP and ERp44 in nuclear Ca^{2+} transient in pCMV-IRES-RED (control) and pCMV-ERp44-IRES-RED (ERp44 over-expressed) HeLa cells. To measure nuclear Ca^{2+} transient x-t images were collected by drawing a line crossing the nucleus region (Figure 2a). The results demonstrated that after application of ATP caused a huge Ca^{2+} transient in the control cells (Figure 2b left and 2c). Whereas, the peak of Ca^{2+} transient induced by extracellular ATP greatly reduced in ERp44 over-expressed cells (Figure 2b right and 2c).

To test if the nuclear Ca^{2+} transients caused by extracellular ATP were mediated through IP_3Rs as reported in cytoplasm, the pCMV-IRES-RED and pCMV-ERp44-IRES-RED cells were cultured with 2-APB, a cell-permeable IP_3Rs inhibitor, 100 μmol/L for 30 min before the experiments. As shown in Figure 2c the amplitude of nuclear Ca^{2+} transient stimulated by ATP was almost completely abolished by 2-APB in both cell types, suggesting that the extracellular ATP activation and ERp44 inhibition of nuclear calcium transient are mediated by IP_3Rs.

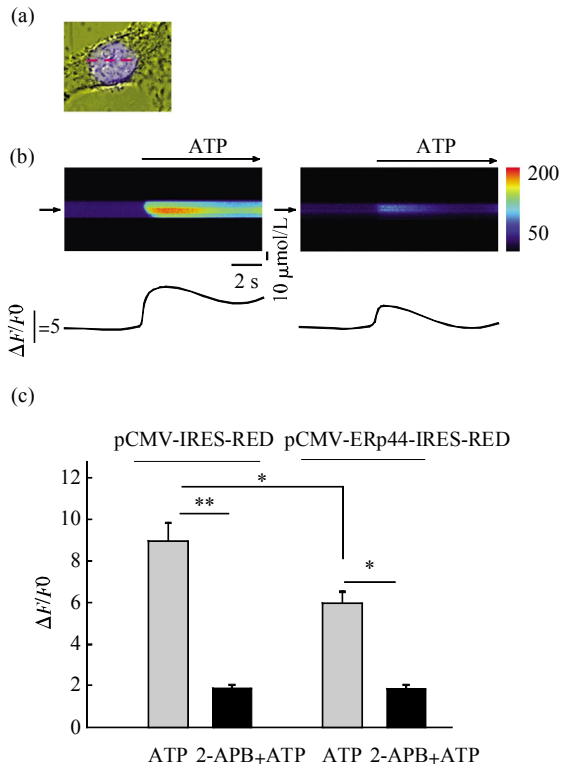

Fig. 2　ERp44 inhibits extracellular ATP-induced nuclear calcium transient

(a) A representative of experiments shows the place where the line scan images were collected. Note, the dotted line was drawn only cross the nucleus. (b) Representative samples of line scan images collected from a pCMV-IRES-RED cell (left) and a pCMV-ERp44-IRES-RED cell (right); the lower panels are fluorescence profiles taken from the above images as indicated by arrows. (c) Summary data of the amplitude of nuclear Ca^{2+} transients (n = 64～82 cells, *P < 0.05 and **P < 0.001), respectively.

2.3　Nuclear IP_3Rs and ERp44 regulated CREB phosphorylation

Previous studies have shown that nuclear calcium transient could induce phosphorylation of the transcription factor cAMP response element binding protein (P-CREB) and subsequently activation of its transcription [17, 24–26]. In the present study, by using Western blotting, the roles of extracellular ATP and ERp44 in regulation in CREB phosphorylation and expression were examined in both pCMV-IRES-RED and pCMV-ERp44-IRES-RED cells. The results indicated that extracellular ATP significantly enhanced CREB phosphorylation compared to control in pCMV-IRES-RED cells, and that this effect of extracellular ATP on CREB phosphorylation was greatly reduced by pre-treatment of cell with 2-APB. As expected, the enhancement of CREB phosphorylation by extracellular ATP was significantly inhibited by over-expression of ERp44 in HeLa cells (Figure 3), however. Similarly, pre-treatment of the pCMV-ERp44-IRES-RED cells with 2-APB, CREB phosphorylation induced by extracellular ATP was reduced, further.

As shown in Figure 3a, extracellular ATP had no significant effect on the expression level of CREB in both cell types.

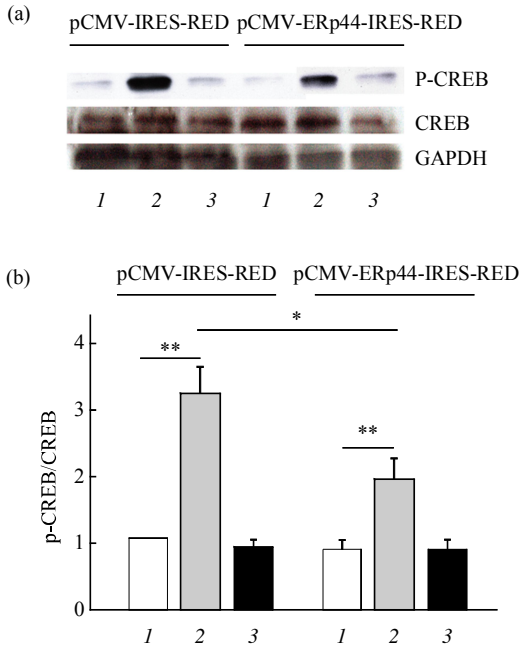

Fig. 3　Effects of ERp44 protein on nuclear IP_3Rs induced CREB phosphorylation

(a) HeLa cells transfected with pCMV-IRES-RED (control) and pCMV-ERp44-IRES-RED (ERp44) were cultured with ATP 10 μmol/L for 2 min or ATP + 2-APB 100 μmol/L for 30 min; the total proteins were then collected and Western blotting was applied. ATP increased the CREB phosphorylation in both cell types, the increases in the CREB phosphorylation induced by ATP were blocked by 2-APB and significantly diminished by the over-expression of ERp44. (b) Summary data of ratio of p-CREB/CREB (n =4, *P < 0.05 and **P < 0.001), respectively. *1*: Control; *2*: ATP; *3*: ATP+2-APB.

2.4　Extracellular ATP and ERp44 regulated gene c-Myc expression

C-Myc is a transcription factor and potent stimulator of cell growth[27–29]. It has been reported that calcium signals trigger the expression of c-Myc [17]. In order to know what effects of extracellular ATP and ERp44 on the expression of c-Myc, we designed and

conducted the real-time PCR experiments in present study. The results demonstrated that extracellular ATP induced a significant increase in the expression of c-Myc in pCMV-IRES-RED cells, and pre-treatment of the cells with 2-APB reduced the expression level of c-Myc (Figure 4) significantly. The ATP activated expression of cMyc was markedly inhibited by the over-expression of ERp44 in HeLa cells (Figure 4).

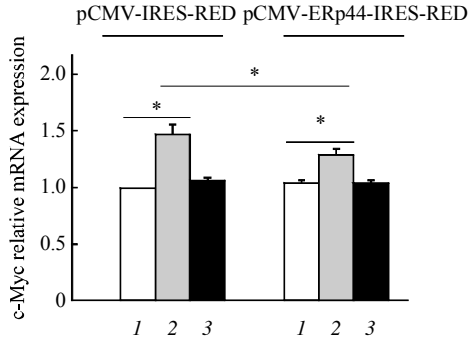

Fig. 4 Extracellular ATP induced but ERp44 inhibited c-Myc gene expression in HeLa cells

HeLa cells transfected with pCMV-IRES-RED (control) and pCMV-ERp44-IRES-RED (ERp44) was stimulated with ATP 10 μmol/L for 30 min alone, or co-pre-treaded with ATP and 2-APB 100 μmol/L for 30 min; total mRNA was collected for real-time PCR. ATP activated expression of c-Myc in both cell types. The activation of c-Myc expression was blocked in the presence of 2-APB. Compared to the control, over-expression of ERp44 reduced expression of c-Myc. $n=4$, $^*P<0.05$. *1*: Control; *2*: ATP; *3*: 2-APB+ATP.

3 Discussion

It has been commonly believed that ERp44 is an ER luminal protein and plays an important role in protein folding and thiol-mediated retention [13–14]. Recent studies have shown that ERp44 is the first identified regulatory protein of IP_3R_1 function from the ER lumen side [15]. As an ER luminal protein of the thioredoxin family, ERp44 directly interacts with the third lumenal loop of IP_3R_1 and that the interaction is dependent on pH, Ca^{2+} concentration and redox state[15]. However, the roles of ERp44 in regulating nuclear calcium release and subsequent gene transcription have not been investigated, yet.

In the present study, HeLa cells have been employed to examine whether the nuclear calcium release is induced by extracellular ATP through IP_3Rs and is regulated by ERp44. Firstly, we demonstrated that only IP_3Rs were present in HeLa cells (Figure 1a), which was consistent with previously published results [15, 22]; the distribution of IP_3Rs and ERp44 displayed a very nice co-localization on the ER and nucleus membranes (Figure 1b). Secondly, we showed that extracellular ATP induced a significant nuclear calcium release *via* IP_3Rs; the peak of the calcium releases induced by extracellular ATP was significantly reduced by over-expression of ERp44, and was almost entirely abolished by pre-treated cells with 2-APB (Figure 2). 2-APB has been widely used as a cell-permeable IP_3R inhibitor though several recent reports demonstrate that this compound can also inhibit store-operated Ca^{2+} channels (SOCs), activate temperature-activated transient receptor potential ion channels (TRPs) and Orai3 [30–33]. 10 μmol/L ATP was used to induce Ca^{2+} release because this concentration of ATP was the minimum one that could induce functional and unsaturated calcium transient in our present study, which was also used by other groups [15]. Thirdly, our findings demonstrated that extracellular ATP and ERp44 regulated CREB phosphorylation through IP_3Rs mediated Ca^{2+} release (Figure 3, 4). It is well known that phosphorylation of Ser133 of CREB specifically enhances its transactivation [29]. Ours and other group′s studies show a similar result [16–18], namely, increase in the nuclear Ca^{2+} content leads to an enhancement of the phosphorylation of CREB. c-Myc, a transcription factor, is a potential and important stimulator for cell growth[28–29]. The expression level of c-Myc could be up-regulated by many activators, including Ca^{2+}. Interestingly, in the present study we find also that ERp44 protein exhibits an inhibitory effect on the expression of c-Myc in HeLa cells(Figure 4), and this effect of ERp44 on c-Myc expression is achieved by the decrease of nuclear Ca^{2+} content *via* the inhibition of IP_3Rs on nuclear membrane.

In summary, our study reveals that extracellular ATP can activate gene transcription *via* IP_3Rs located on nuclear membrane and ERp44 displays a down-regulation of gene transcription in HeLa cells.

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内质网蛋白 44(ERp44)通过 1, 4, 5-三磷酸肌醇受体介导基因转录 *

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摘要　细胞核内钙离子浓度的增加可以引起包括钙离子激活的基因转录在内的很多生理功能．运用 Western blot、免疫荧光、实时定量聚合酶链反应、钙成像以及外源三磷酸腺苷刺激细胞释放钙离子等试验方法，发现 1, 4, 5- 三磷酸肌醇受体和内质网蛋白 44(ERp44)在内质网和核膜上都有很好的共定位．外源三磷酸腺苷可以通过 1, 4, 5- 三磷酸肌醇受体刺激核内钙瞬变并磷酸化环磷酸腺苷反应原件结合蛋白(CREB)、刺激原癌基因 c-Myc 的表达．但是，这些功能都能被 1, 4, 5- 三磷酸肌醇受体抑制剂 2- 氨乙氧基二苯酯硼酸(2-APB)和过表达内质网蛋白 44(ERp44)所抑制．这些结果均提示在子宫颈癌 HeLa 细胞中内质网蛋白 44(ERp44)通过 1, 4, 5- 三磷酸肌醇受体而介导基因转录．

关键词　内质网蛋白 44(ERp44)，三磷酸腺苷，1, 4, 5- 三磷酸肌醇受体，钙瞬变，基因转录

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