



Original Article

Rat mammary epithelial cells line: A biphasic activation of extracellular signal-regulated kinase (ERK) 1/2

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ABSTRACT

Animal models help scientists evaluate the molecular basis of cancer and explore new treatment options. ERK 1/2, a cell signaling component, is a critical molecule in pathogenesis and an important target for breast cancer. In the brain and some other tissues, ERK 1/2 phosphorylation shows a biphasic time-dependent activation. We hypothesize that ERK 1/2 phosphorylation exhibits a similar pattern in rat breast cell lines. The rat mammary epithelial cells (RMECs) were isolated and exposed to RPMI 1640 containing 5%, 10%, and 15% rat serum, with serum-free media as a negative control. Using the WST-1 technique, we evaluated cell proliferation. The cells were exposed to 10% serum-enriched media, and the cell lysate was treated with RIPA buffer in a time-dependent manner (1, 5, 10, 15, and 30 minutes). ERK 1/2 phosphorylation was evaluated by Western blot using antibodies against total and phosphorylated rat ERK 1/2 proteins. The cell proliferation assay showed that serum at a concentration over 5% can induce cell proliferation in RMECs. Serum media at concentrations higher than 10% had the same effect on cell proliferation. ERK1/2 proteins were phosphorylated 5 minutes after exposure, underwent dephosphorylation after 10 minutes, and rephosphorylation occurred from 15 to 30 minutes as a second phase. Taken together, we can infer that the behavior of cell signaling components, especially the ERK1/2 phosphorylation pattern, differs from species to species.

سلول‌های اپیتلیال پستان موش صحرایی: فعال‌سازی دوفازی کیناز تنظیم‌شده با سیگنال خارج سلولی ERK 1/2

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چکیده

مدل‌های حیوانی به دانشمندان کمک می‌کند که اساس مولکولی سرطان را ارزیابی کرده و گزینه‌های درمانی جدید را کشف کنند. ERK 1/2، یک جزء سیگنال دهنده سلولی، یک مولکول حیاتی در پاتوژنز و یک هدف مهم برای سرطان سینه است. در مغز و برخی بافت‌های دیگر، فسفوریلاسیون ERK 1/2 یک فعال‌سازی دوفازی وابسته به زمان را نشان می‌دهد. ما فرض می‌کنیم که فسفوریلاسیون ERK 1/2 الگوی مشابهی را در رده‌های سلولی سینه موش نشان می‌دهد. سلول‌های اپیتلیال پستان موش (RMECs) جدا شده و در معرض RPMI 1640 حاوی ۵، ۱۰ و ۱۵ درصد سرم موش، با محیط‌های بدون سرم به عنوان کنترل منفی قرار گرفتند. با استفاده از تکنیک WST-1، تکثیر سلولی را ارزیابی کردیم. سلول‌ها در معرض محیط‌های ۱۰ درصد غنی شده با سرم قرار گرفتند و لیزات سلولی با بافر RIPA به روشی وابسته به زمان (۱، ۵، ۱۰، ۱۵ و ۳۰ دقیقه) تیمار شد. فسفوریلاسیون ERK 1/2 توسط وسترن بلات با استفاده از آنتی بادی بر علیه پروتئین کل و فسفریله موش ERK 1/2 مورد بررسی قرار گرفت. سنتجش تکثیر سلولی نشان داد که سرم در غلظت بیش از ۵ درصد می‌تواند تکثیر سلولی را در RMEC ها القا کند. محیط‌های سرمی در غلظت‌های بالاتر از ۱۰ درصد تأثیر یکسانی بر تکثیر سلولی داشتند. پروتئین‌های ERK1/2 5 دقیقه پس از قرار گرفتن در معرض فسفریله شدند، پس از ۱۰ دقیقه تحت دفسفوریلاسیون قرار گرفتند و فسفوریلاسیون مجدد از ۱۵ تا ۳۰ دقیقه به عنوان فاز دوم رخ داد. روی هم رفته، می‌توانیم استنباط کنیم که رفتار اجزای سیگنال‌دهنده سلول، به‌ویژه الگوی فسفوریلاسیون ERK1/2، از گونه‌ای به گونه دیگر متفاوت است.

واژه‌های کلیدی: موش صحرایی، دوفازی، ERK 1/2، فسفوریلاسیون

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INTRODUCTION

Animal models have a long history, and the rat model is used for several studies [1]. Breast cancer animal models help scientists evaluate the molecular basis of cancer and explore new treatment options [2]. However, there are some doubts regarding the use of these models because they cannot exactly mimic human breast cancer [3]. Cell signaling pathways are some of the most important targets for breast cancer treatment because the role of these proteins is well documented [4]. Thus, the concordance of these signaling pathways in human and rat models should be evaluated. About 30% of human breast cancers harbor mutations in the ERK1/2 pathway, which belongs to the mitogen-activated protein kinases [5]. There are four types of these kinases, including ERK1/2, ERK5, JNK, and p38 [6]. The ERK1/2 proteins, alongside other signaling pathways, change the gene expression profile, ultimately leading to breast cancer cell transformation and proliferation. The activation of the ERK1/2 cascade is initiated via growth factors, cytokines, and hormones through tyrosine kinase receptors, G protein-coupled receptors, and non-nuclear receptors of steroid hormones [7]. After the activation of Ras, Raf is activated, which in turn causes the phosphorylation of MEK. Finally, MEK activates the ERK1/2 protein by phosphorylation on serine and threonine residues [8]. Activated ERK1/2 exerts its effects by phosphorylating several substrates in the cytoplasm, cell membrane, and nucleus. The downstream substrates are kinases that extend the upstream effects. In this kinase cascade, there are many substrates for ERK1/2, which is why this signaling is crucial in breast cancer development [9]. The rat model is used to study breast cancer, and the results are applied to humans. Animal experiments on this protein can be valid only if the activation and deactivation

of this pathway are in concordance with human and animal models. In this study, we will investigate the phosphorylation of this pathway in the rat model by isolating rat mammary tissue cells and exposing them to activating factors.

MATERIALS AND METHODS

Isolation RMECs populations

After presenting the research proposal, the ethics committee of Islamic Azad University, Urmia Branch, issued the ethics code (<https://ethics.research.ac.ir/IR.IAU.URMIA.REC.1402.112>). We isolated RMEC populations based on a previously reported protocol. The digestion procedure and characterization of RMECs with forward scatter and side scatter were performed [10]. Rat mammary epithelial cells were separated from hematopoietic and endothelial cells based on the lack of surface antigen.

WST-1

In sterile 96-well plates, 10,000 cells/well were seeded, and serum-starved medium and serum-enriched media with 0.5%, 10%, and 15% were added to the cultures for 24 hours. Using WST-1 tetrazolium, the surviving cells were determined [11]. The optical density (OD) was measured at 490 nm, with a reference wavelength of 630 nm for each well, and the OD of serum-enriched wells was divided by the OD of serum-free wells.

Treatment and protein extraction

The cells were treated with RPMI 1640 containing 10% rat serum, separated from a normal rat. Five treated flasks were incubated with lysis buffer after 1, 5, 10, 15, and 30 minutes of exposure to serum-enriched media.

The control group received serum-free RPMI 1640 culture media. The media were removed and washed once with PBS, and the proteins were extracted with RIPA lysis buffer containing antiprotease. The total protein sample levels were measured with the Bradford test and a standard concentration of albumin [12].

SDS PAGE and western blot

The protein samples were applied to the wells and separated by the SDS-PAGE technique. The gel was blotted onto a PVDF membrane. Then the membrane was blocked with 3% BSA in TBST for 2 hours. Incubation with anti-rat ERK1/2 and anti-phospho-rat ERK1/2 antibodies (ERK 1/2 Antibody (MK1): sc-13590) was performed overnight at 4°C. Horseradish peroxidase-conjugated anti-mouse IgG secondary antibody was used to complete the tests. After each step, PVDF membranes were washed with TBST and TBS buffers. Visualization of bands was performed via enhanced chemiluminescence.

Statistical Analysis

Data analysis was performed using SPSS. After

conducting the normality test, the mean of the variable was compared between groups using ANOVA or non-parametric tests, depending on the data distribution. A p-value less than 0.05 was considered statistically significant.

RESULTS

Cell proliferation assay

The results showed that serum at a concentration over 5% is able to induce cell proliferation in RMECs. There was a significant difference in cell proliferation between the cells treated with 5% serum-enriched media and serum-free media. As shown in Figure 1, serum media above 10% had the same effect on cell proliferation.

ERK1/2 phosphorylation

The effect of 10% serum-enriched media on the phosphorylation of ERK1/2 showed that the proteins were phosphorylated 5 minutes after exposure and underwent dephosphorylation after 10 minutes. Rephosphorylation occurred from 15 to 30 minutes as a second phase. The total ERK1/2 levels were equal in all samples.

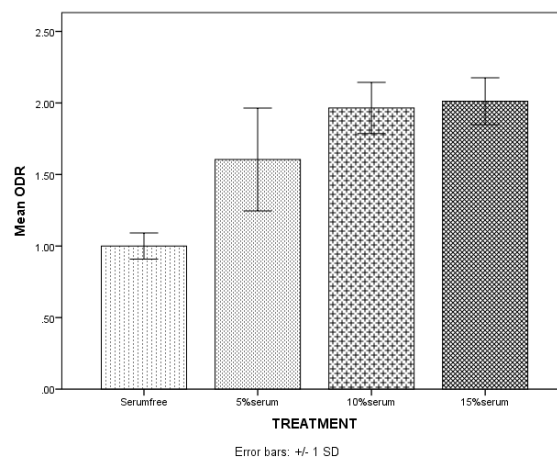


Figure 1. The effect of serum enrichment on cell proliferation; 5%, 10%, and 15% serum enrichment increased cell proliferation. The 10% concentration had no significant difference from 15% in proliferative effects.

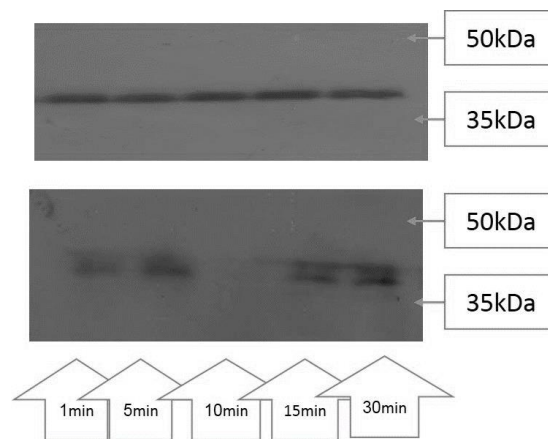


Figure 2. Time-dependent activation of ERK1/2 phosphorylation at 5 minutes and dephosphorylation after 10 minutes of exposure.

DISCUSSION

Animal models can be an informative source of experiments if they have a similar molecular signature to the human entity. The evaluation of rat genetics was completed in 2004, but the proteomics, especially cell signaling components, need further investigation [13]. Here, we evaluated the serum effect on cell proliferation in rat mammary epithelial cells. Rat serum was used to minimize the immunogenic reaction. Serum contains a plethora of growth factors that can induce cell proliferation, and most of them exert their effects via ERK1/2. The concentration assessment showed that 10% serum enrichment could induce cell proliferation, and higher concentrations had a non-significant difference. Thus, we used 10% serum as the optimum concentration. The effect of serum enrichment on cell proliferation has been reported by others [14, 15]. We observed time-dependent phosphorylation of ERK1/2 signaling occurring within the first hour. This protein is activated in human cell lines within the first hour. For example, EGF leads to ERK1/2 phosphorylation in corneal cells in less than five minutes [16]. There is no doubt that ERK1/2 phosphorylation is a process that occurs in less than one hour in almost all cell lines. It should be noted that the activation of this signaling could affect the activation of other signaling pathways. For

example, activation of ERK1/2 delays the activation of the Akt signaling pathway [12]. We observed biphasic phosphorylation of ERK1/2 in RMECs. Trifilieff et al. demonstrated that ERK1/2 activation has a biphasic pattern in brain tissue [17]. ERK1/2 phosphorylation showed biphasic activation in retinal pigment epithelium cells [18]. Here, two key points should be considered. First, the phosphorylation of ERK1/2 has biphasic activation in normal rat mammary cells, and the behavior of the protein is not completely indicative for human studies.

CONCLUSION

Taken together, we can infer that the behavior of cell signaling components depends on the species from which the cell is isolated. The ERK1/2 phosphorylation pattern varies from cell to cell and species to species. Therefore, human cells isolated from patients are the best tools for signaling pathway evaluation, especially for the ERK1/2 protein.

ETHICS

Approved.

CONFLICT OF INTEREST

None.

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