Role of the Rho/Rho-kinase Pathway in Endometrial Adenocarcinoma Cell Proliferation

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ABSTRACT

Present study investigated the possible role of the Rho/ROCK pathway in the proliferation of endometrial adenocarcinoma HEC-1A cells. Cell proliferation was monitored using the xCELLigence system. HEC-1A cells were incubated with a ROCK inhibitor, Y-27632 (10^{-7}–10^{-5} M) in the presence and absence of the RhoA activators, lysophosphatidic acid (LPA; 10^{-6} M) and calpeptin (10^{-6} M). The cell index was evaluated after incubation for 60, 72, 84, 96, and 108 h. Both LPA and calpeptin significantly increased cell proliferation at 60–108 h, which could be reversed by treatment with 10^{-5} M (but not 10^{-7} or 10^{-6} M) Y-27632. On the other hand, Y27632 (10^{-7}–10^{-5} M) alone increased cell proliferation at all the time points tested. Conclusion: The Rho activators, LPA and calpeptin, induced cell proliferation in HEC-1A cells at least in part via ROCK. ROCK inhibitor, Y-27632, reversed the effects of LPA and calpeptin and increased the cell index. Further studies are required to investigate this.

Keywords: Endometrial adenocarcinoma, HEC-1A, Rho/ROCK pathway, Proliferation

ÖZET

Endometrial Adenokarsinoma Hücre Proliferasyonunda Rho/Rho-Kinaz Yolağının Rolü

Bu çalışma, Rho/ROCK yolağının endometrial adenokarsinom HEC-1A hücrelerinin proliferasyonundaki olası rolünü araştırmıştır. Hücre proliferasyonu, xCELLigence sistemi kullanılarak izlendi. HEC1A hücreleri, Rho-kinaz (ROCK) inhibitörü, Y-27632 (10^{-7}-10^{-5} M) ile, RhoA aktivatörleri, lisofosfatidik asit (LPA, 10^{-6} M) ve calpeptin(10^{-6} M) varlığında ve yokluğunda inkübe edildi. Hücre indeksi, 60, 72, 84, 96 ve 108 saatlik inkübasyonlarda değerlendirildi. Bulgular: Hem LPA hem de calpeptin, proliferasyonu 60-108 saatler arasında anlamlı şekilde arttırdı. 10^{-5} M Y-27632 (fakat 10^{-7} ve 10^{-6} M değil) LPA ve calpeptinin proliferatif etkisini tersine çevirdi. Öte yandan, Y27632 (10^{-7}-10^{-5} M) tek başına test edilen tüm zaman noktalarında hücre çoğalmasını arttırdı. Rho aktivatörleri, LPA ve calpeptin, HEC1A hücrelerinde en azından kısmen Rho-kinaz yoluyla hücre proliferasyonunu indüklemiştir. ROCK inhibitörü, Y-27632, LPA ve calpeptinin etkisini tersine çevirdi, ayrıca bu inhibitör, tek başına hücre indeksinde bir artış sağladı. Bu durum daha fazla araştırmayı gerektirmektedir.

Anahtar Kelimeler: Endometrial adenokarsinoma, HEC 1A, Rho/ROCK yolağı, Proliferasyon

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INTRODUCTION

Endometrial carcinomas are the fourth most common malignancy in females, after lung, breast, and colorectal cancers.[1] They predominantly originate from the uterine epithelium (90%), and 75%-80% are adenocarcinomas.[2] Various intracellular signaling mechanisms are involved in the proliferation of endometrial adenocarcinomas. For example, protein kinase B (Akt) signaling has been shown to enhance cell proliferation in endometrial adenocarcinomas via cyclooxygenase 2 enzyme.[3,4] "Cross-talk" between mitogen-activated protein kinase signaling and estrogen receptors has also been shown to be involved, and activation of this pathway may affect proliferation of endometrial cancer.[5] It was reported that activation of STAT3 and ERK1/2 signaling was involved in cancer growth in Ishikawa endometrial cancer cells.[6] It was also shown that hepatocyte growth factor has chemotactic and antiapoptotic effects in RL95-2 endometrial carcinoma cells, and enhances cellular infiltration via the PI3K/Akt pathway.[7] On the other hand, protein kinase C has been reported to play a tumor suppressor role in the endometrium.[8] Thus, regulating the receptors and intracellular signaling pathways initiated by activation of protein kinase C may represent a new therapeutic target in cancer. Several antineoplastic drugs that inhibit multiple protein kinases are currently in use and under developement.[9] One of the most important cellular signaling pathways in cancer is the Rho/Rho-kinase (Rho/ROCK) pathway.[10] The small G protein, Rho, and its downstream effector, ROCK, contribute to various cellular events such as actin reorganization, cell migration and adhesion, cell cycle regulation, gene expression, and apoptosis.[11] RhoA is the most studied subtype of Rho protein and there is much evidence showing that RhoA may interact with the downstream effector ROCK, which may also be important in cancer growth, invasion, and metastasis.[12] In addition, Rho GTPs have been reported to play a role in Ras-mediated oncogenic transformation.[13] However, members of Rho family, including RhoA, RhoC, RhoH, Cdc42, and Rac1, have been shown to be overexpressed in some types of cancer.[12,14,15] Inhibition of the Rho/ROCK pathway has been shown to attenuate tumor invasion and metastasis, both in vivo and in vitro. For example, MM1 rat hepatoma cells expressing a structurally active ROCK mutation showed increased invasive activity, which was suppressed by the ROCK inhibitor, Y-27632, leading to reduced peritoneal spread of the cells.[16] In addition, Y-27632 was reported to inhibit the invasive ability of Walker 256 carcinosarcoma, N1E-115 neuroblastoma, human hepatocellular carcinoma, and PC3 human prostate cancer cells.[11,17-19] Another ROCK inhibitor, Wf-536, was reported to inhibit invasion of human HT1080 fibrosarcoma and B16 murine melanoma cells.[20,21] Furthermore, the ROCK inhibitor, fasudil, was shown to reduce tumor progression in human and rat models.[22] It was also reported that RhoA is overexpressed in breast tumors.[14] Lysophosphatidic acid (LPA) is a cell membrane lipid that plays an important role in tumor invasion and metastasis in many types of cancer, enhancing secretion/activation and transcription of matrix metalloproteinase-7 via LPA receptor 2 (LPAR2).[23,24] Consequently, LPA appears to play a potential regulatory role in the proliferation and invasion of malignant cells.

On the other hand, the Rho-kinase inhibitor, fasudil, decreased cell proliferation, DNA synthesis, and G2/M and S phase cell number of endometrial stromal cells.[25]

Taken together, the Rho/ROCK signaling pathway substantially contributes to cancer cell proliferation. However, the effect of RhoA and its downstream effector ROCK on tumor cell proliferation in HEC-1A endometrial adenocarcinoma cells and its effects on tumor progression remain unclear. The HEC-1A endometrial adenocarcinoma cell line is widely used due to primary cell isolation and characterization difficulties.[26] Therefore, the present study investigated the possible contribution of the Rho/ROCK pathway to proliferation of endometrial adenocarcinoma cells.

MATERIALS AND METHODS

This study was conducted between May 2015 and November 2015.

Cell Culture

The human endometrial adenocarcinoma HEC-1A cell line was purchased from American Type Cul-

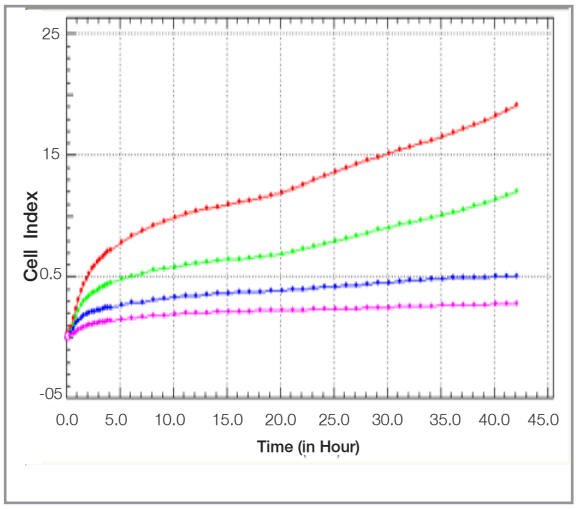

Figure 1. Real-time monitoring of cell proliferation. HEC1A cells were seeded at 2500, 5000, 10000 and 20000 cells/well in E-plate (down to up, respectively) (n= 4).

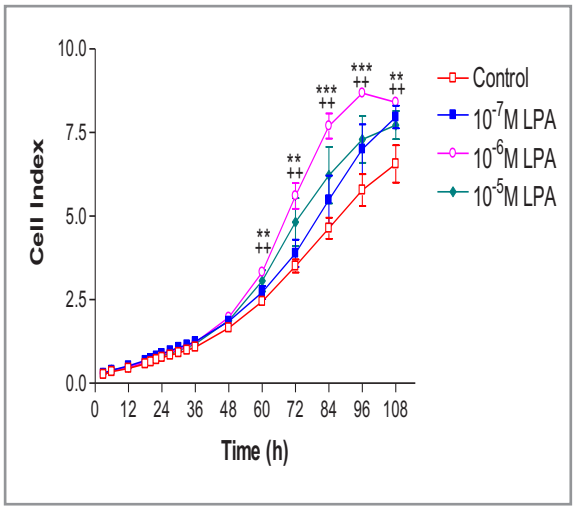

Figure 2. The effect of LPA on the proliferation of HEC 1A cells. 10^{-6} and 10^{-5} M LPA significantly increased proliferation at 60., 72., 84., 96. and 108. h (LPA= Lysophosphatidic acid, *= Control vs 10^{-6} M LPA, += Control vs 10^{-5} M LPA) (n= 4).

ture Collection (ATCC HTB112 ATCC, Manassas, VA) and maintained in a humidified atmosphere incubator at 37^0C with 5% CO_2. HEC-1A cells were grown in modified McCoy's 5A medium (Lonza) containing 10% fetal bovine serum (FBS; Gibco).

Proliferation Assay

HEC-1A cells were seeded in E-plates at densities of 2500, 5000, 10000 and 20000 cells per well and monitored in real-time using the xCELLigence system (Roche) to determine the ideal cell number titration for subsequent experiments. The ideal cell number was determined to be 10000. The impedance value of each well was automatically monitored using the xCELLigence system and expressed as a cell index (CI) value. A total of 10000 cells were seeded per well and incubated with Rho activators, calpeptin (10^{-4}–10^{-6} M) and oleyl L-α-lysophosphatidic acid sodium salt (10^{-5}–10^{-7} M). In another group of experiments, cells were incubated with ROCK inhibitor, Y-27632 (10^{-5}–10^{-7} M), in the presence and absence of calpeptin (10^{-6} M) and LPA (10^{-6} M). Control cells were incubated with drug vehicles. CI values were evaluated after incubation for 60, 72, 84, 96, and 108 h.

Drugs

Calpeptin was purchased from Cytoskeleton Inc. (Denver, CO, USA), Y-27632 from Tocris Cookson (Bristol, UK.), and oleyl L-α-lysophosphatidic acid sodium salt from Sigma Aldrich (Saint Louis, USA). Oleyl L-α-lysophosphatidic acid sodium salt was dissolved in phosphate-buffered saline (PBS) in the presence of 0.1% (w/v) bovine serum albumin. A stock solution of calpeptin was dissolved in DMSO and diluted with PBS. The final concentration of DMSO in the plates did not exceed 1/10000 (v/v).

Statistical Analysis

Data were expressed as mean ± standard error of observations. For comparison, analysis of variance followed by Bonferroni's multiple comparison test was used. P-values less than 0.05 were considered significant. GraphPad Prism 3, GraphPad Software (San Diego, CA, USA), was applied in performing the statistical analyses.

RESULTS

Cell Number Titration

To determine the optimum cell number for subsequent experiments, cells were seeded in E-plates

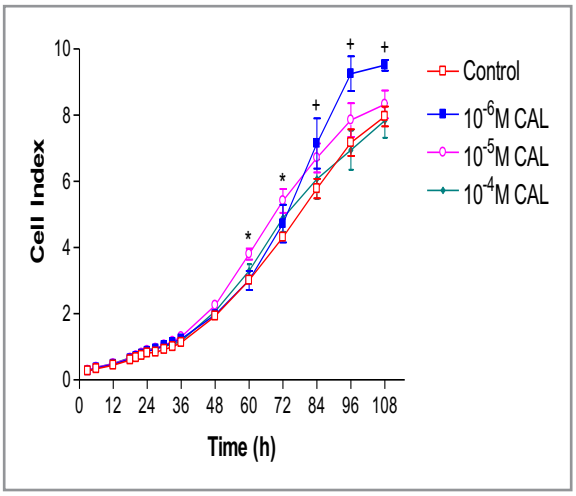

Figure 3. The effect of calpeptin on the proliferation of HEC 1A cells. 10^{-6} M at 84, 96 &108 h and 10^{-5} M at 60 h & 72h increased the proliferation (CAL:Calpeptin, *= Control vs 10-6 M CAL, += Control vs 10-5 M CAL). (n= 4).

at densities of 2500, 5000, 10000, and 20000 cells per well (n= 4). Cells seeded at a density of 10000 cells per well showed an increase in CI compatible with a cell doubling time of around 31 h (Figure 1). Therefore, 10000 cells/well were used for subsequent experiments.

Effect of RhoA activator, LPA, on HEC-1A cell proliferation

LPA increased endometrial adenocarcinoma HEC-1A cell proliferation. The effect of LPA (10^{-5}–10^{-7}) observationally started at 48 h, but statistical significance was first observed at 60 h. Concentrations of 10^{-6} and 10^{-5} M LPA significantly increased proliferation at 60, 72, 84, 96, and 108 h; however, 10^{-7} M LPA showed no statistically significant increase on cell proliferation at any time point. Although 10^{-6} and 10^{-5} M LPA were equally effective on cell proliferation, we chose to use the lower concentration (10^{-6} M LPA) in subsequent experiments (n= 4 each) (Figure 2).

Effect of RhoA activator, calpeptin, on HEC-1A cell proliferation

Another well-known RhoA activator, calpeptin, showed no effect on cell proliferation at 10^{-4} M, but increased cell proliferation at 10^{-6} M (at 84, 96, and 108 h) and 10^{-5} M (at 60 and 72 h). The statistical significance more pronounced for 10^{-6} M calpeptin; therefore, subsequent experiments used this concentration (n= 4 each) (Figure 3).

Effect of ROCK inhibitor, Y27632, on LPA-induced HEC-1A cell proliferation

Since 10^{-6} M LPA significantly increased HEC-1A cell proliferation at 60, 72, 84, 96, and 108 h, subsequent experiments were conducted using this concentration of LPA. Cells were treated with 10^{-6} M LPA in the absence and presence of ROCK inhibitor Y27632 (10^{-7}–10^{-5} M). Y27632 (10^{-7}–10^{-6} M) did not reverse LPA-induced cell proliferation, whereas 10^{-5} M Y27632 decreased cell proliferation at 60, 84, and 96 h. In contrast, Y27632 (10^{-7}–10^{-5} M) alone significantly increased HEC-1A cell proliferation (n= 6) (Figure 4).

Effect of ROCK inhibitor, Y27632, on calpeptin-induced HEC-1A cell proliferation

HEC-1A cells were treated with 10^{-6} M calpeptin since this concentration was the most effective concentration that induced proliferation. Cells were treated with 10^{-6} M calpeptin in the absence and presence of ROCK inhibitor, Y27632 (10^{-7}–10^{-5} M). Similar to the effects observed for LPA, 10^{-5} M Y27632 reversed the effect of calpeptin (at 72, 84, 96, and 108 h). However, lower concentrations of Y27632 (10^{-6}–10^{-7} M) showed no effect on calpeptin-induced cell proliferation (n= 6) (Figure 5). Both the reversal effect on LPA-induced HEC-1A cell proliferation and the proliferative effect pattern of Y27632 were confirmed in calpeptin-treated cells (Figures 4 and 5).

DISCUSSION

The present study investigated the potential role of the Rho/ROCK pathway in the proliferation of endometrial adenocarcinoma cells. The Rho activators, LPA and calpeptin, increased the proliferation of endometrial adenocarcinoma HEC-1A cells. The ROCK inhibitor, Y27632 (10^{-5} M), reversed the effect of both LPA and calpeptin. On the other hand, treatment with Y27632 (10^{-7}–10^{-5} M) alone

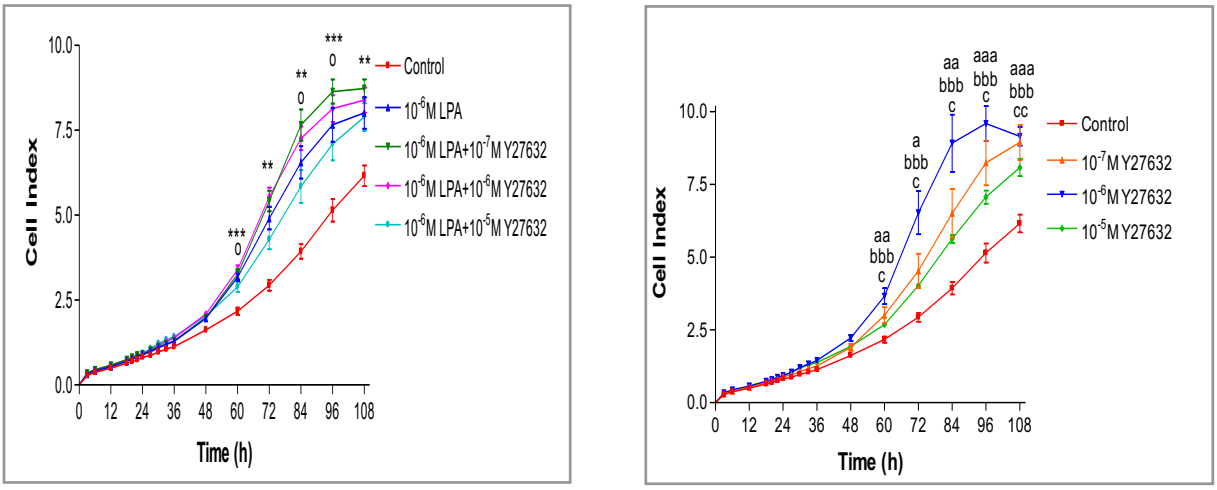

Figure 4. The effect of ROCK inhibitor Y27632 on the LPA induced proliferation of HEC 1A cells. 10^{-5} M Y27632 reversed the LPA induced cell proliferation but 10^{-7}-10^{-6} M did not. Y27632 alone (10^{-7}-10^{-5}) significantly increased the proliferation of HEC1A cells. (LPA= Lysophosphatidic acid, *= Control vs 10^{-6} M LPA, o= LPA vs LPA+10^{-5} M Y27632, a= Control vs 10^{-7} M Y27632, b= Control vs 10^{-6} M Y27632, c= Control vs 10^{-5} M Y27632 (n= 6).

increased the proliferation of endometrial adenocarcinoma HEC-1A cells.

While the role of the Rho/ROCK pathway in the proliferation of many types of cancers has been extensively studied,[10,27-31] the effect of this pathway on the proliferation of endometrial adenocarcinoma HEC-1A cells has not been reported.

LPA receptors, especially LPAR1 and LPAR2, have been shown to be overexpressed in endometrial cancer compared with non-cancerous endometrial tissue.[32] In addition, LPA induces in vitro proliferation and invasion of endometrium adenocarcinoma HEC-1A cells, and LPAR2 has been shown to mediate LPA-induced endometrial cancer invasion.[23] In the present study, we also observed proliferative effects of LPA in HEC-1A cells, which is compatible with the findings of these previous studies. The effect of LPA observationally started at 48 h, but statistical significance was first observed after 60 h. All our proliferation experiments were performed in medium containing 10% FBS. Our results may have been influenced by the presence of FBS, which may have affected cell proliferation. It is possible that LPA and calpeptin-induced proliferation may have been greater and seen earlier in the absence of FBS.

A previous study reported the preventive effect of calpeptin on the proliferation of malignant pleural mesothelioma cells; however, few studies have reported its effects on cancer cell proliferation, and none have investigated its effects on endometrial adenocarcinoma HEC-1A cell proliferation.[33] The present study is the first to demonstrate that calpeptin significantly increases the proliferation of endometrium adenocarcinoma HEC-1A cells.

The effect of ROCK on tumor cell proliferation is heterogeneous. ROCK is reported to have a positive effect on tumor growth, and ROCK inhibitors are known to decrease tumor cell proliferation in various tumor tissues, such as acute myeloid leukemia, non-small cell lung cancer, ovarian cancer, prostate cancer, and breast cancer.[28,34-38] However, some studies have reported no effect of these inhibitors on cell proliferation.[39,40] On the other hand, studies have shown that ROCK inhibitors increased cell proliferation in colon cancer and pancreatic cancer cells.[29,30] Consistent with these studies, we found that Y27632 increased proliferation of HEC-1A cells. However, higher (10^{-5} M), but not lower (10^{-7} and 10^{-6} M), concentrations of Y27632 were able to reverse the proliferation promoting effects of both calpeptin and LPA on HEC-1A cells. LPA is known to activate both RhoA and STAT3 protein, and promote colorectal carcinoma cell proliferation via activation of these two proteins.[41] A similar situation may exist in endometrial adenocarcinoma HEC-1A cells, and LPA may ac-

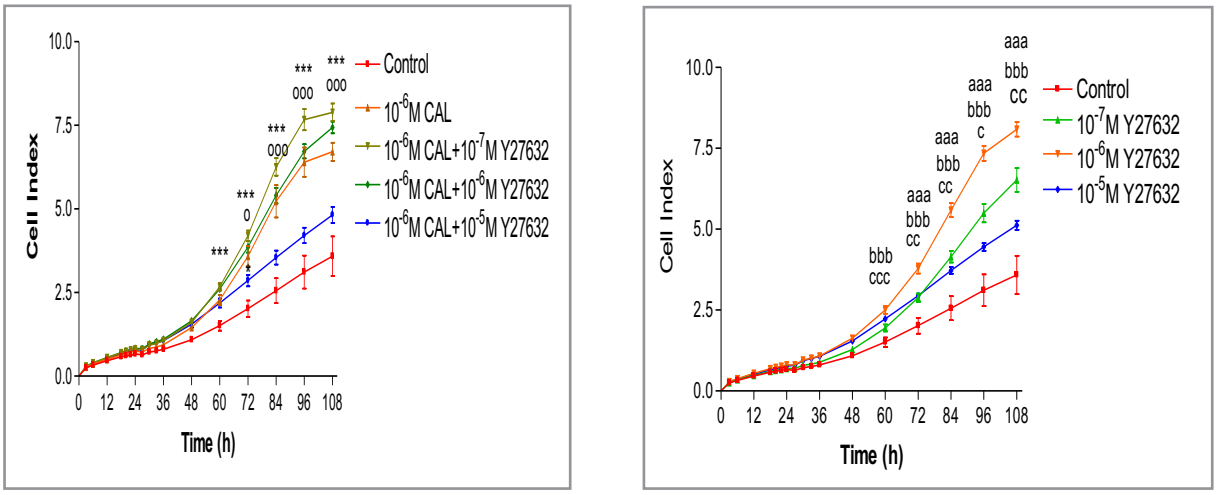

Figure 5. The effect of ROCK inhibitör Y27632 on the calpeptin induced proliferation of HEC 1A cells. 10^{-5} M Y27632 revesed the effect of calpeptin but 10^{-7}-10^{-6} M did not. Y27632 alone (10^{-7}-10^{-5}) significantly increased the proliferation of HEC1A cells. CAL= Calpeptin, *= Control vs 10^{-6} M CAL, o= CAL vs CAL +10^{-5} M Y27632, a= Control vs 10^{-7} M Y27632, b= Control vs 10^{-6} M Y27632, c= Control vs 10^{-5} M Y27632. (n= 6)

tivate other proteins that play a role in cell proliferation. However, ROCK inhibitor alone could not sufficiently reverse the effects of LPA.

Interestingly, Y-27632 was shown to have dual activity on the proliferation of these cells. On its own, Y27632 (10^{-7}–10^{-5} M) increased the cell proliferation. This inhibitor had differential effects in the presence and absence of RhoA activators, which it may be a nonspecific effect that requires further investigation.

CONCLUSION

The Rho activators, LPA and calpeptin, were shown to increase HEC-1A cell proliferation. This effect was at least in part via ROCK, and treatment with the ROCK inhibitor, Y-27632 alone, increased cell proliferation. These findings require further investigation.

Acknowledgments

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