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# Dexamethasone inhibits U937 cell adhesion *via* the down-regulation of ROCK1 activity

Dong Liu#, Xing-Yun Chen#, Ren-Ping Xiong, Ya-Lei Ning, Ping Li, Yan Peng, Ping Liu, Yan Zhao, Nan Yang and Yuan-Guo Zhou✉

Molecular Biology Center, State Key Laboratory of Trauma, Burn, and Combined Injury, Research Institute of Surgery and Daping Hospital, Third Military Medical University, Chongqing 400042, China

**Objective. To explore the effect of dexamethasone (DEX) on monocyte adhesion function and its underlying mechanism. Methods. The effects of DEX and fasudil on adhesion of cultured U937 monocytes to human umbilical vein endothelial cells (HUVEC) following stimulation with phorbol myristate acetate (PMA) were studied; Changes in the Rho-associated coiled-coil protein kinase 1 (ROCK1) protein content and activity were evaluated. Results. DEX and fasudil significantly inhibited U937 cell adhesion rates under PMA stimulation and inhibited ROCK1 activity. Mifepristone (RU-486) and cycloheximide (CHX) did not alter these effects of DEX. Conclusions. DEX interferes with the adhesion function of U937 cells through the inhibition of ROCK1 activity.**

**Key words**: dexamethasone, fasudil, ROCK1, non-genomic effects

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## INTRODUCTION

The monocyte–macrophage system plays a critical role in acute inflammation reactions, such as acute lung injury and systemic inflammatory response syndrome. A significant increase in monocyte-macrophage adhesion capacity is an important basis of the inflammatory cascade (Clark, 2007). Glucocorticoids are known to have strong anti-inflammatory effects through non-genomic and genomic effects (Clark, 2007). However, the mechanism of monocyte-macrophage adhesion inhibition by glucocorticoids is not clear. Previous studies have shown that Rho-associated coiled-coil protein kinase 1 (ROCK1) play an important role in inflammatory reaction, and it can be selectively inhibited by treatment with fasudil (Slotta *et al.,* 2006). Here we tested the adhesion capacity of U937 monocyte-macrophage cells and intracellular ROCK1 activity to explore the mechanism of inhibition of excessive activation of macrophages by corticosteroids in acute inflammatory responses.

## MATERIALS AND METHODS

**Materials**. Human monocyte-macrophage cell line U937 and human umbilical vein endothelial cells (HUVEC) were stored in liquid nitrogen at the Molecular Biology Center, Research Institute of Surgery and Daping Hospital, Third Military Medical University. Phorbol myristate acetate (PMA) solution: PMA powder (Sigma) was dissolved in dimethylsulfoxide (DMSO) to a concentration of 100 μg/ml, aliquoted into sterile tubes, and stored in the dark at –20°C. Powders of dexamethasone (DEX), mifepristone(RU-486), and cycloheximide (CHX, Sigma) were dissolved at $1 \times 10^{-4}$ M in ethanol, aliquoted, and stored at 4°C. Fasudil was purchased from Asahi Kasei Pharma Corporation, dissolved in saline at 10 mM, aliquoted, and stored at 4°C. Cell Counting Kit-8 (CCK-8, Bi Yun Tian Biotechnology Research Institute China) was stored in the dark at 4°C. A 0.25% of trypsin solution was purchased from Gibco, aliquoted, and stored at –20°C. Culture plates (24- and 96-well) were purchased from Nunclon Company. Mouse anti-human ROCK1 antibody was purchased from BD Biosciences, rabbit anti-human activated ROCK1 1113/1114 antibody was purchased from Abcam Inc., and goat anti-mouse/rabbit secondary antibody was obtained from Sigma.

**Methods. Cell culture**. Cryopreserved U937 cells were recovered and cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS), and HUVEC cells were cultured in DMEM medium (Gibco) containing 10% FBS. The cells were cultured in an incubator at 37°C with 5% $CO_2$.

**Measurement of U937 and HUVEC adhesion rates**. After the digestion of HUVEC cells, which were grown under optimal conditions, with 0.25% trypsin, DMEM medium containing 10% FBS was used to adjust the density to $4 \times 10^4$ cells/ml. Aliquots of 100 μl were added to each well of a 96-well plate and placed in a 37°C, 5% $CO_2$ incubator for 8–14 hours. After the cells had grown and adhered to the well to form a single layer, the original medium was removed and the cells were washed twice with serum-free medium. U937 cells at a density of $5 \times 10^5$ cells/ml were added to the wells at 100 μl per well. Six experimental groups were used: control, 400 ng/ml PMA, 400 ng/ml PMA + $10^{-6}$ M DEX, 400 ng/ml PMA + $10^{-6}$ M DEX + $10^{-4}$ M RU-486, 400 ng/ml PMA + $10^{-6}$ M DEX + $10^{-4}$ M CHX, and 400 ng/ml PMA + 100 μM fasudil. The 96-well plate was placed in the cell incubator for 1 hour. The medium was then discarded, and the cells were gently washed twice with sterile PBS solution to remove the U937 cells that were not adhered. After adding 90 μl of PBS and 10 μl of a 10% CCK-8 solution to each well, the plate was placed in the cell incubator for 1–2 hours. OD values were then measured using a microplate reader at 450 nm. OD values were compared between the groups.

✉e-mail: ygzhou@cta.cq.cn; yuanguo.zhou@gmail.com
#These authors contributed equally to this work

**Abbreviations**: CCK-8, Cell Counting Kit-8; CHX, cycloheximide; DEX, Dexamethasone; DMSO, dimethylsulfoxide; HUVEC, human umbilical vein endothelial cells; PMA, phorbol myristate acetate; ROCK1, Rho-associated coiled-coil protein kinase 1; RU-486, mifepristone.

**Western blot detection of ROCK1 protein expression and its activity.** After the extraction of total cellular protein using a conventional method, the Bradford method was used to determine its concentration (Compton *et al.,* 1985). Then 30 μg of total protein boiled with 2× loading buffer and subjected to 7.5% SDS/PAGE with uniform sample volumes per well. Proteins were wet-transferred to a PVDF membrane, and after blocking with 3% BSA for 5 h at room temperature, mouse anti-human ROCK1 primary antibody (1:500) and rabbit anti-ROCK1 cleavage site 1113/1114 primary antibody (1:1000), which was used to evaluate ROCK1 activity indirectly, were added. The membrane was subsequently incubated at room temperature for 2 h. Then HRP-labeled goat anti-mouse/rabbit secondary antibody (1:5000) was added. The results were recorded with X-ray film after adding enhanced chemiluminescence reagent (ECL, GE Healthcare). Labworks 4.6 image analysis software was used to analyze the intensity of band with β-actin as an internal control. The experiments were repeated three times (Liu *et al.,* 2008).

**Statistical analysis**. The experimental results were expressed as the mean ± standard deviation ($X \pm S$). Sigmaplot 11.0 and SPSS 17.0 were used for graph construction and statistical analysis, respectively. A single-factor analysis of variance was used for multiple group comparisons. A value of $P<0.05$ was considered statistically significant.

## RESULTS

### Changes in the U937-HUVEC cell adhesion capacity

After PMA stimulation, the U937 cell adhesion rate was significantly increased relative to the non-stimulated group ($P<0.01$). After the addition of DEX or fasudil, the adhesion rate was significantly lower than in the PMA stimulation group ($P<0.01$). Furthermore, the addition of RU-486 or CHX did not block the DEX inhibition of PMA stimulation ($P>0.05$).

### Changes in ROCK1 protein expression

The total content of ROCK1 protein in U937 cells did not change significantly following the addition of various agents.

### Changes in ROCK1 activity

After the addition of PMA, ROCK1 activity was significantly increased relative to the control group ($P<0.05$). After the addition of inhibitors, such as DEX and fasudil, the ROCK1 activity decreased significantly when compared to the PMA stimulation group ($P<0.05$). The addition of glucocorticoid receptor antagonist RU-486 or the protein synthesis inhibitor CHX did not affect the DEX inhibition of ROCK1 activity ($P>0.05$).

## DISCUSSION

Glucocorticoids play a crucial role in anti-inflammatory responses and immune suppression. In addition to the well-known long-term effects of glucocorticoids, there are also other pathways, such as non-genomic effects, that are known to have rapid actions. Such rapid actions do not require gene transcription and protein synthesis, and thus the reaction time is short, and the response is efficient (Strehl *et al.,* 2011; Zen *et al.,* 2011).

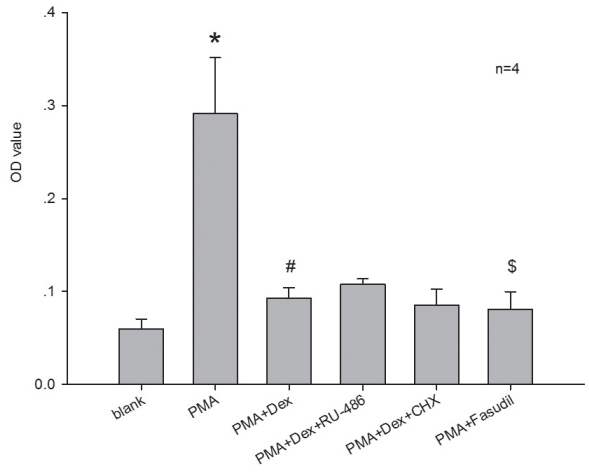
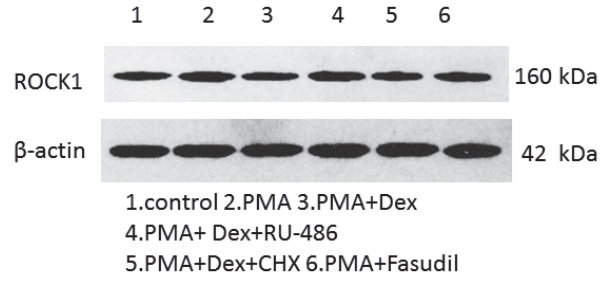



**Figure 1. U937 cell adhesion to HUVEC cells**
Note: When compared to the blank control group, $^{*}P<0.05$; when compared with the PMA-stimulated group, $^{\#}P$ and $^{\$}P$ were both $<0.05$.n=4.

This study found that DEX inhibition of U937 cell adhesion was not affected by the addition of the classic glucocorticoid receptor antagonist RU-486 or the addition of the protein synthesis inhibitor CHX. This result indicated that the DEX suppression of the U937 adhesion mechanism may function *via* non-genomic effects. This study found that the use of fasudil could effectively reduce the adhesion function of macrophages. Fasudil, as a specific inhibitor of ROCK, a n important downstream effector molecule of the small G-protein Rho signal-



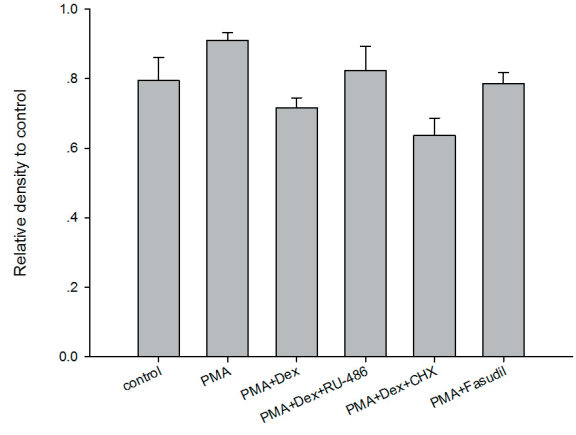




**Figure 2. ROCK1 protein content following various treatments.**
**Top**, a representative of Western blot. **Bottom**, quantitative data for ROCK1 protein expression. Difference are not statistically significant.

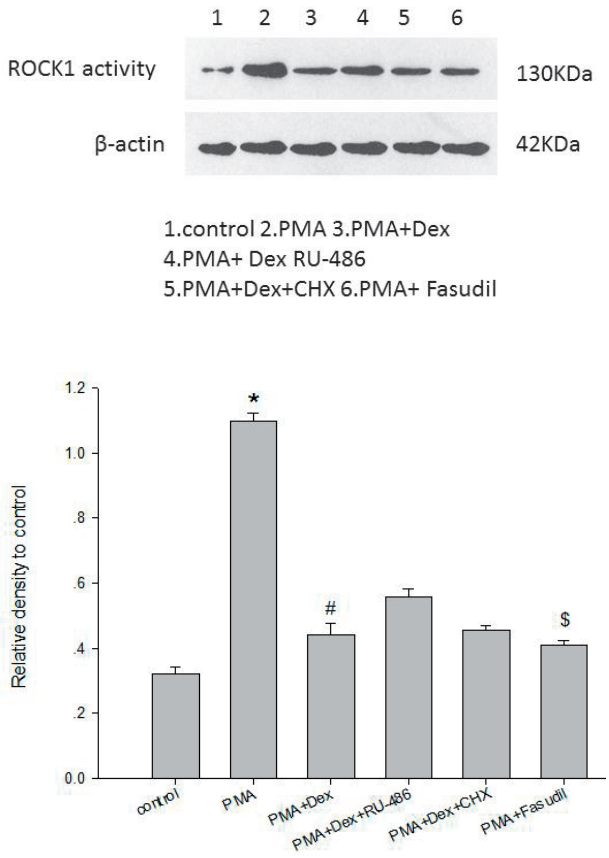



**Figure 3. ROCK1 activity content following various treatments. Top**, a representative of Western blot. **Bottom**, quantitative data for ROCK1 activity expression. Note: When compared to the blank control group, $^{*}P<0.05$; when compared to the PMA-stimulated group, $^{\#}P$ and $^{\$}P$ were both <0.05.

ing pathway, has been used for treatment of a variety of diseases associated with ROCK (Riento *et al.,* 2003; Surma *et al.,* 2011). The experimental results presented here suggest that ROCK may play an important role in the adhesion mechanism of macrophages. In the human, ROCK exists as two subtypes, ROCK1 and ROCK2, of similar structures. However, ROCK1 is mainly distributed in the lungs, liver and kidneys, whereas ROCK2 is mainly found in the brain (Nakagawa *et al.,* 1996). An increasing number of experiments have shown that the roles of these two subtypes of ROCK are not similar and may even be mutually antagonistic. Currently, an obvious distinction between the specific roles of ROCK1 and ROCK2 is not known. Studies have shown that ROCK1 plays a major role in the neutrophil adhesion process, whereas ROCK2 does not (Kume, 2008). In a study of lipopolysaccharide-induced acute lung injury in mice, lung inflammation was significantly reduced after giving fasudil (Li *et al.,* 2010). In addition, an experimental analysis of coronary artery ligation-induced vascular injury also confirmed that ROCK1 was the main cause of neutrophil aggregation and vascular injury (Noma *et al.,* 2008). Therefore, the inhibition of ROCK1 expression may be the key to suppressing inflammation. In the experiments presented here, the mechanism of macrophage adhesion inhibition by DEX was similar to that of fasudil, thereby suggesting that DEX may also interact with ROCK. Our experiments found that in both the PMA stimulation group and the DEX and fasudil treatment group, the ROCK1 protein content showed no obvious change. However, in the DEX and fasudil treatment group, ROCK1 activity was significantly reduced relative to the PMA stimulation group. Therefore, we speculate that DEX and fasudil have similar actions, namely the inhibition of ROCK1 activity, thereby inhibiting cell adhesion, which does not affect the ROCK1 protein content.

## CONCLUSION

Although the role of glucocorticoids in the inhibition of acute inflammation and of allergic reactions, and in immunosuppression has undisputed therapeutic potential, accompanying side effects have restricted its use. The mechanisms of the genomic and non-genomic effects of glucocorticoids have gradually been discovered. Experiments have confirmed that glucocorticoids affect the cell adhesion junction complex *via* RhoA (Rubenstein *et al.,* 2003), although it remains unclear whether glucocorticoids affect ROCK1. The experimental results presented here suggested that DEX inhibited the activation of ROCK1 and thus hindered the adhesion function of monocyte-macrophage cells, which occurred as a non-genomic effect. In other words, ROCK1 may be an important effector molecule of glucocorticoids in a non-genomic effect pathway. In the course of using glucocorticoids to inhibit the over-adhesion of inflammatory cells, future work will be necessary to improve the specific effects of the glucocorticoid treatment while highlighting its impact on ROCK1 inhibition and avoiding the occurrence of other genomic and non-genomic effects. In conclusion, through the measurement of U937 monocyte-macrophage cell adhesion, this study found that DEX suppresses the up-regulation of U937 cell adhesion under PMA stimulation by inhibiting ROCK1 activity. However, the specific molecular mechanism by which DEX inhibits ROCK1 activity remains unclear and requires further study.

### Conflict of Interests

The authors declare that they have no conflict of interests.

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## REFERENCES

Clark AR (2007) Anti-inflammatory functions of glucocorticoid-induced genes. *Mol Cell Endocrinol* **275**: 79–97.

Compton S, JJones CG (1985). Mechanism of dye response and interference in the Bradford protein assay. Anal Biochem **151**: 369–374.

Kume H (2008) RhoA/Rho-kinase as a therapeutic target in asthma. *Curr Med Chem* **15**: 2876–2885.

Li Y, Wu Y, Wang Z, Zhang X, HWu WK (2010) Fasudil attenuates lipopolysaccharide-induced acute lung injury in mice through the Rho/Rho kinase pathway. *Med Sci Monit* **16**: BR112–BR118.

Liu X, Li P, Liu P, Xiong R, Zhang E, Chen X, Gu D, Zhao Y, Wang Z, Zhou Y (2008) The essential role for c-Ski in mediating TGF-beta1-induced bi-directional effects on skin fibroblast proliferation through a feedback loop. *Biochem J* **409**: 289–297.

Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S (1996) ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett* **392**: 189–193.

Noma K, Rikitake Y, Oyama N, Yan G, Alcaide P, Liu P Y, Wang H, Ahl D, Sawada N, Okamoto R, Hiroi Y, Shimizu K, Luscinskas

FW, Sun J, Liao JK (2008) ROCK1 mediates leukocyte recruitment and neointima formation following vascular injury. *J Clin Invest* **118**: 1632–1644.

Riento K, Guasch RM, Garg R, Jin B, Ridley AJ (2003) RhoE binds to ROCK I and inhibits downstream signaling. *Mol Cell Biol* **23**: 4219–4229.

Rubenstein NM, Guan Y, Woo P L, Firestone GL (2003) Glucocorticoid down-regulation of RhoA is required for the steroid-induced organization of the junctional complex and tight junction formation in rat mammary epithelial tumor cells. *J Biol Chem* **278**: 10353–10360.

Slotta JE, Braun OO, Menger MD, Thorlacius H (2006) Fasudil, a Rho-kinase inhibitor, inhibits leukocyte adhesion in inflamed large blood vessels *in vivo*. *Inflamm Res* **55**: 364–367.

Strehl C, Gaber T, Lowenberg M, Hommes DW, Verhaar AP, Schellmann S, Hahne M, Fangradt M, Wagegg M, Hoff P, Scheffold A, Spies CM, Burmester GR, Buttgereit F (2011) Origin and functional activity of the membrane-bound glucocorticoid receptor. *Arthritis Rheum* **63**: 3779–3788.

Surma M, Wei LShi J (2011) Rho kinase as a therapeutic target in cardiovascular disease. *Future Cardiol* **7**: 657–671.

Zen M, Canova M, Campana C, Bettio S, Nalotto L, Rampudda M, Ramonda R, Iaccarino LDoria A (2011) The kaleidoscope of glucorticoid effects on immune system. *Autoimmun Rev* **10**: 305–310.