Role of RHOB in the antiproliferative effect of glucocorticoid receptor on macrophage RAW264.7 cells

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Abstract

Although glucocorticoid (GC) has been reported to inhibit macrophage killing activity and cytokine production in response to proinflammatory stimuli, the effect of GC on macrophage proliferation is controversial. In our previous study, we found that inhibition of glucocorticoid receptor (GR) expression in murine macrophage cell line RAW264.7 cells (RAW-GR(−) cells) by RNAi significantly promoted cell proliferation. In the present study, we provide the evidence that the expression of *Rhob*, a member of Rho GTPases with anti-cancer character, remarkably decreased in RAW-GR(−) and RAW264.7 cells transiently transfected with GR-RNAi vector. Overexpression or constitutive activation of *Rhob* in RAW-GR(−) and RAW264.7 cells by transfection with wild-type *Rhob* expression vector (*Rhob*-wt) or constitutively activated *Rhob* plasmid (*Rhob*-V14) resulted in decreased proliferation of the two cell lines. Oppositely, the proliferation of RAW264.7 cells was

significantly increased when the expression of *Rhob* by RNA interference technique or the activity of *Rhob* by transfection with dominant negative *Rhob* mutant that is defective in nucleotide binding (*Rhob*-N19) was inhibited. In addition, enhanced activity of Akt, but not MAPK3/1 or MAPK14, was found in RAW-GR(−) cells. Blocking the pathway of phosphatidylinositol 3-kinase (PI3K)/Akt with the specific inhibitor LY294002 decreased the proliferation and elevated RHOB protein level, indicating that PI3K/Akt signal plays its role of proliferation modulation upstream of RHOB protein. In conclusion, these results demonstrate that *Rhob* plays an important role in the antiproliferative effect of GR on RAW264.7 cells by GR → Akt → *Rhob* signaling and *Rhob* negatively regulates the proliferation of RAW264.7 cells.

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Introduction

Glucocorticoid (GC) is a significant hormone in regulating diverse physiological processes such as immune and inflammation responses, cell proliferation, differentiation, and apoptosis (Dondi *et al.* 2001, Kudawara *et al.* 2001, Distelhorst 2002, Yamamoto *et al.* 2002). The common use of GC in treating inflammatory and autoimmune diseases is mostly due to its function of immunosuppression, for instance, inhibition of growth and induced apoptosis in lymphocytes, and repression of cytokine production in macrophages (Distelhorst 2002, Frankfurt & Rosen 2004). Macrophages also play a key role in the immune and inflammatory process, mainly acting as antigen-processing/presenting cells and sources of inflammatory cytokines. Although GC has been reported to suppress macrophage killing activity and cytokine production in response to proinflammatory stimuli (Brummer *et al.* 2005), the effect of GC on macrophage proliferation is controversial (Lloberas *et al.* 1998, Sakai *et al.* 1999, Pagniello *et al.* 2002).

It is well known that the action of GC was mediated by glucocorticoid receptor (GR) that belongs to nuclear receptor

superfamily and is normally localized in the cytoplasm in an inactive state. On hormone binding, GR translocates to the nucleus where it binds to glucocorticoid response elements (GREs) on target genes, recruits coactivators, or corepressors to regulate the expression of target genes (Heitzer *et al.* 2007). But a few target genes have been known to mediate the antiproliferative effect of GC until now. *Rhob* is a member of Rho GTPase family and has a tumor-suppressive role, including inhibiting cell proliferation and inducing apoptosis in several human cancer cells, and inhibiting tumor growth in a nude mouse xenograft model (Prendergast 2001, Mazieres *et al.* 2004). *Rhob* expression level has recently been shown to dramatically decrease in several cancer cells and patient biopsies such as lung cancer, brain cancer, neck and head tumors as tumors become more aggressive, and ectopic expression of *Rhob* antagonizes oncogene-mediated transformation (Liu *et al.* 2001, Adnane *et al.* 2002, Forget *et al.* 2002, Jiang *et al.* 2004a,b, Mazieres *et al.* 2004, Huang & Prendergast 2006). Recently, we found that GC/GR increased *Rhob* expression in several tumor cells, such as human ovarian cancer cell lines HO-8910 and 3AO, as well as human hepatocellular carcinoma cell

SMMC-7721, and the proliferation inhibition of GC/GR on HO-8910 cells is partly due to the increased expression of *Rhob* (Chen *et al.* 2006). Although *Rhob* activation was required for mannose receptor-mediated phagocytosis of human alveolar macrophages (Zhang *et al.* 2005), the role of *Rhob* in the proliferation of macrophage was unclear until now.

In our previous study, we found that Dex inhibited the growth of RAW264.7 cells, a murine macrophage cell line. Treatment of RAW264.7 cells with 10^{-7} M Dex for 4 days resulted in about 27% inhibition of cell proliferation. We also found unexpectedly that inhibition of GR expression in RAW264.7 by RNA interference significantly promoted cell proliferation. The cell number of RAW-GR(–) cells (stably transfected with GR RNA interference plasmid) was almost twofold as that of the RAW-control cells (stably transfected with RNA interference control plasmid) after 4-day culture (Zhu *et al.* 2004). These results indicated that GC/GR negatively regulated the growth of RAW264.7 cell, and compared with GC, the expression of functional GR seemed to be more important in keeping cells a more differentiated and less proliferative phenotype. This prompted us to further clarify the molecular mechanism responsible for the antiproliferative effect of GR on RAW264.7 cells. In this study, we investigated the expression of *Rhob* in RAW-GR(–) cells and its possible role in GC/GR-mediated proliferation inhibition of RAW264.7 cells.

Materials and Methods

Materials

Materials used in the present study included mouse monoclonal antibodies against β -actin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma–Aldrich Chemicals); rabbit polyclonal antibodies against GR and *Rhob* (Santa Cruz Biotechnology, Santa Cruz, CA, USA); goat anti-rabbit-AP and goat anti-mouse-AP conjugate (Bio-Rad Laboratories); RPMI 1640 medium (Life Technologies Inc.); RNA interfering expression vector pSilencer 2.1-U6 and negative control plasmid (Ambion, Austin, TX, USA); and BCA protein assay kit (Pierce, Pierce, IL, USA).

The wild-type *Rhob* expression vector (pcDNA3-*Rhob*; named *Rhob*-wt), constitutively active *Rhob* mutant containing a point mutation in the GTPase domain (G14V; named *Rhob*-V14), and dominant negative *Rhob* mutant that is defective in nucleotide binding (T19N; named *Rhob*-N19) were generously provided by Dr Prendergast (2001).

Cell lines and cell culture

Murine macrophage cell line RAW264.7 was obtained from American Type Culture Collection (Rockville, MD, USA). RAW-GR(–) and RAW-control cells were established by stable transfection of RAW264.7 cells with GR-siRNA expression vector and siRNA control vector respectively (Zhu *et al.* 2004). All the cells were cultured in RPMI 1640

medium containing 10% heat-inactivated fetal bovine serum at 37 °C in a humidified incubator of 5% CO₂.

Quantitative real-time PCR

Total RNA was extracted with the TRIzol reagent and 2 μ g total RNA was used to synthesize first-strand cDNA by reverse transcription system (Promega), in accordance with the manufacturer's instructions.

Primers for amplification of *Rhob* gene were sense: 5'-CTG GCC CGC ATG AAG CA-3' and antisense: 5'-AGG CAG TCT GGT GGT GTC C-3' (186 bp), and those for β -actin were sense: 5'-CTG TAT GCC TCT GGT CGT AC-3' and antisense: 5'-TGA TGT CAC GCA CGA TTT CC-3' (214 bp). SYBR Green from OPEN company was used as the fluorescence reagent. cDNA template (100 and 50 ng for the amplification of *Rhob* and β -actin respectively) was added to the mixture of 2 μ l 10 $\times$ buffer, 1.6 μ l of 25 mM MgCl₂, 0.5 μ l of 20 mM dNTP, 0.3 μ l of 20 mM primers, 1.5 U *Taq* enzyme, and 1.0 μ l of 20 $\times$ SYBR Green to a final volume of 20 μ l. Amplification of *Rhob* and β -actin was performed in different tubes, with the same process: denaturation at 94 °C for 15 s, annealing at 57 °C for 20 s, elongation at 72 °C for 15 s for 36 cycles, and a final extension for 10 min at 72 °C.

The mRNA ratio of *Rhob* and actin was calculated as follows: we first drew the standard curves of *Rhob* and β -actin by measuring the threshold cycles (CT) of standards of different concentrations, which had strong positive relationship with the start copy number (i.e. cDNA amounts) of a gene in linear amplification period. Then the cDNA amounts of *Rhob* and β -actin of each testing sample were calculated according to their standard curves, and cDNA amount of *Rhob* was normalized by the corresponding β -actin.

Western blot analysis

Whole cell extract was prepared with lysis buffer (Stamatakis *et al.* 2002; 10 mM Tris (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 0.5% SDS, 0.1 mM mercaptoethanol, 2 μ g/ml of each of the protease inhibitors leupeptin, aprotinin, and pepstatin). The lysate was briefly sonicated on ice, and spun at 14 000 r.p.m./min for 10 min to remove the insoluble material. Protein concentrations of the supernatant were measured with BCA protein assay kit. Twenty to fifty micrograms of extracts were resolved on 10% SDS–PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, which were then blocked with 5% nonfat milk and probed overnight with antibodies against *Rhob* (1:200), GR (1:1000), total and phosphorylated MAPK3/1, MAPK14, phosphorylated Akt (1:500), or β -actin (1:10 000). Then the membranes were washed, exposed to alkaline phosphatase (AP)-conjugated secondary antibodies for 2 h, and finally detected by NBT/BCIP staining. The quantitiveness of protein expression was made by Tanon Bio-image software (Shanghai, China).

RNA interference constructs for *Rhob* and transfection

Using online siRNA sequence selector tool (BD Clontech), siRNA templates were designed to match nonconserved 19 nucleotide sequences within the mouse *Rhob* mRNA. The sequences of the oligonucleotides used to create *Rhob*-RNAi were: 1) sense: 5'-GAT CCC GCC TAT GAC TAC CTC GAG TTT CAA GAG AAC TCG AGG TAG TCA TAG GCT TTT TTG GAA A-3' and antisense: 3'-GGC GGA TAC TGA TGG AGC TCA AAG TTC TCT TGA GCT CCA TCA GTA TCC GAA AAA A CC TTT TCG A-5', and 2) sense: 5'-GAT CCC GCG CTA CGG ATC CCA GAA TTT CAA GAG AAT TCT GGG ATC CGT AGC GCT TTT TTG GAA A-3' and antisense: 3'-GGC GCG ATG CCT AGG GTC TTA AAG TTC TCT TAA GAC CCT AGG CAT CGC GAA AAA ACC TTT TCG A-5'. Negative control plasmid was supplied from Ambion; it expressed a hairpin siRNA with limited homology to any known sequences in the human, mouse, and rat genomes. The oligonucleotides were synthesized by Sangon Technology Inc. (Shanghai, China) and were annealed/cloned into pSilencer 2.1-U6 vectors. The clones were confirmed by DNA sequencing. Expression of siRNA for *Rhob* is under control of the U6 promoter.

pSilencer 2.1-U6-*Rhob* or pSilencer 2.1-U6 control plasmid was introduced into RAW264.7 cells with LipofectAMINE Plus reagent (Invitrogen). Briefly, the cells were transfected with 3 µg DNA in each well of a 6-well plate, according to the manufacturer's instructions.

Transient transfection of the cells

Cells were seeded onto 24-well plates (4×10^4 cells/well) or 6-well plates (2×10^5 cells/well) for 24 h and then transiently transfected with 1 (for 24-well plate) or 3 µg (for 6-well plate) of the blank vector (pcDNA3), *Rhob*-wt, *Rhob*-V14, *Rhob*-RNAi, or *Rhob*-N19 plasmid using LipofectAMINE Plus reagent (Invitrogen), according to the manufacturer's instructions.

Cell proliferation assay

Twenty-four hours after transfection with various *Rhob* plasmid as indicated above, cells were rinsed out and seeded in duplicate at 3×10^4 cells per well onto 24-well plate for MTT assay or 2×10^5 cells per well onto 6-well plate for cytometry. After incubation for 2 days, the cell number in each well was collected and counted with a hemacytometer or examined by MTT assay. Two hundred microliters of RPMI 1640 medium containing 5 mg/ml MTT (Sigma-Aldrich Chemicals) solution were added to each well. The cells were incubated for another 4 h at 37 °C, followed by solubilization with 200 µl DMSO solutions. Then the O.D. value was measured using a Bio-Rad (model 550) microplate reader at 550 nm with the DMSO as blank.

The linear response range of RAW264.7 cells between the results of MTT assay and the number of cells was explored, and it was found that there was a linear correlation when the cells were seeded at the densities from 1×10^4 to 4×10^5 cells per well onto a 24-well plate and cultured in the medium for 4 days (data not shown). Therefore, in our experiments, 3×10^4 cells per well in a 24-well plate were selected, cultured in the medium for 4 days, and then cell proliferation was detected by MTT assay.

Statistical analysis

Data are expressed as $X \pm S.D.$ Statistical significance was determined by Student's *t*-test with $P < 0.05$ considered as statistically significant.

Results

Inhibition of GR expression in RAW264.7 cells by stable or transient transfection with GR RNA interference vector decreased Rhob expression of the cells

Considering that *Rhob* showed a proliferation inhibitory effect on several tumor cells, and the proliferation of RAW-GR(−) cells (stably transfected with GR-RNAi vector) increased after inhibition of GR expression, we examined the expression of *Rhob* at mRNA and protein levels in RAW-GR(−) cells by quantitative real-time PCR (Fig. 1A) and western blot analysis (Fig. 1B) respectively. As we expected, the mRNA and protein levels of *Rhob* in RAW-GR(−) cells were reduced to about 22% and 28% of RAW-control cells respectively.

RAW-GR(−) cells were originated from a single clone. Several single clones of RAW-GR(−) cells were established and down-regulation of *Rhob* expression was also found in other clones (data not shown). To validate down-regulation of *Rhob* expression induced by a decreased level of GR, RAW264.7 cells were transiently transfected with GR-RNAi vector and the protein level of *Rhob* was decreased in RAW264.7 cells (Fig. 1C), which indicated that down-regulation of *Rhob* expression in RAW264.7 cells stably or transiently transfected with GR-RNAi vector was indeed a result from the knock down of GR.

Decreased expression of Rhob is involved in the increased proliferation of RAW-GR(−) cell

As increased proliferation was in concurrence with significantly decreased expression of *Rhob* in RAW-GR(−) cells, we hypothesized that decreased expression of *Rhob* might be related to the growth acceleration of RAW-GR(−) cells. To evaluate this possibility, we transfected RAW-GR(−) cells with *Rhob*-wt (*Rhob* wild-type construct) *Rhob*-V14 (a constitutively active *Rhob* plasmid) or blank vector to detect the effect of *Rhob* on the cell proliferation by MTT assay and cytometry. As shown in Fig. 2A, the protein level of *Rhob* increased about twofold in RAW-GR(−) cells transfected with *Rhob*-wt plasmid. The increase in expression and activation

of *Rhob* in RAW-GR(−) cells resulted in about 27 and 34% decrease in cell proliferation respectively (Fig. 2B). Similar results were obtained by cytometry (Fig. 2C). These data indicated that decreased expression of *Rhob* is involved in the increased proliferation of RAW-GR(−) cell.

GC/GR induces the expression of *Rhob* at mRNA and protein levels in RAW264.7 cells

Recently, we found that GC/GR increased *Rhob* expression in human ovarian cancer cell lines HO-8910 and 3AO, as well

as human hepatocellular carcinoma cell SMMC-7721, and the proliferation inhibition of GC/GR on HO-8910 cells is partly due to the increased expression of *Rhob* (Chen *et al.* 2006). To determine the effect of GC on the expression of *Rhob* in RAW264.7 cells, we investigated the mRNA and protein levels of *Rhob* in the cells treated with 10^{-7} M Dex for different times by quantitative real-time PCR and western blot respectively. Figure 3A shows that Dex induced the mRNA expression of *Rhob* in a time-dependent manner, with the maximal effect at 2 h after treatment with 10^{-7} M Dex. A significant increase in RHOB protein expression was also observed following incubation of cells with 10^{-7} M Dex for 4 h. Furthermore, RU486, anti-agonist of GR, could partially attenuate the up-regulation of *Rhob* by Dex, indicating that activation of GR is required for *Rhob* induction (Fig. 3B).

Rhob negatively regulates the proliferation of parental RAW264.7 cells

To examine whether *Rhob* has a direct inhibition effect on the proliferation of parental RAW264.7 cells, we used RNAi (RNA interference) to knock down endogenous *Rhob* expression in this cell line. The results of western blot showed that both the two *Rhob*-RNAi plasmids obviously reduced the expression of *Rhob* in RAW264.7 cells by about 73% [*Rhob*-RNAi(1)] and 88% [*Rhob*-RNAi(2)], when compared with that of control siRNA plasmid (Fig. 4).

The proliferation of RAW264.7 cells was detected by MTT and cytometry after transient transfection with blank vector, *Rhob*-RNAi(2), *Rhob*-N19 (dominant negative mutant plasmid), *Rhob*-wt, or *Rhob*-V14. As shown in Fig. 5A, inhibition of the expression and the activity of *Rhob* by transfection with *Rhob*-RNAi and *Rhob*-N19 resulted in about 44 and 36% increase in the cell number respectively, compared with that of the blank vector control (pcDNA3 or RNAi-control) cells. *Rhob* overexpression or constitutive activation of *Rhob* by transfection of RAW264.7 cells with *Rhob*-wt or *Rhob*-V14 decreased the cell number to about 80 and 62% of the blank

Figure 1 Inhibition of GR expression in RAW264.7 cells by stable or transient transfection with GR RNA interference vector decreased *Rhob* expression of the cells. (A) *Rhob* mRNA level in RAW-GR(−) and RAW-control cells (stably transfected with GR-RNAi vector and RNAi control vector respectively) was detected by quantitative real-time PCR ($X \pm s.d.$, $n=3$). ** $P<0.01$ versus RAW-control. (B) The protein level of RHOB in the two cells was assayed by western blot analysis. Similar results were obtained from three independent experiments. Using Tanon software (Shanghai China), the blots of *Rhob* in these two cells were quantified and normalized against β -actin. The normalized numbers from three independent experiments were compared. The values are expressed as the percentage of the level of RAW-control, as shown in the bar graph of B ($X \pm s.d.$, $n=3$). ** $P<0.01$ versus RAW-control. (C) The protein level of RHOB in RAW264.7 cells transiently transfected with GR-RNAi vector or RNAi control vector was assayed by western blot analysis. Similar results were obtained from three independent experiments.

Figure 2 Transfection of RAW-GR(–) cells with *Rhob* wild-type plasmid or constitutively active *Rhob* plasmid inhibits cell proliferation. (A) The protein level of RHOB in RAW-GR(–) cells was assayed by western blot analysis. Similar results were obtained from three independent experiments. RAW-GR(–) cells were transfected with blank vector, *Rhob*-wt (wild-type *Rhob* cDNA), or *Rhob*-V14 (constitutively active *Rhob* mutant) for 24 h, and then seeded and cultured in duplicate for 2 days onto 24-well plate (3×10^4 cells/well) for (B) MTT assay or (C) 6-well plate (2×10^5 cells/well) for cytometry ($X \pm S.D.$). * $P < 0.05$ versus cells transfected with blank vector. $n = 3$.

vector control cells. Similar results were obtained by MTT assay (Fig. 5B). These results further demonstrated that *Rhob* signaling negatively regulated the proliferation of RAW264.7 cells.

Akt phosphorylation, but not MAPK3/1 and MAPK14, is involved in the accelerating the growth of RAW-GR(–) cells

In recent years, evidence has emerged that GR plays important roles in regulating cell proliferation by modulating

Figure 3 Dex increases *Rhob* expression at mRNA and protein levels in RAW264.7 cells. (A) *Rhob* mRNA expression in RAW264.7 cells after treatment with 10^{-7} M concentrations of Dex for different times was examined by real-time PCR. Dex induced the mRNA expression of *Rhob* in a time-dependent manner, with the maximal effect at 2 h after treatment with 10^{-7} M Dex ($X \pm S.D.$, $n = 3$). ** $P < 0.01$ versus control. (B) Protein level of RHOB in RAW264.7 cells treated with 10^{-7} M Dex with or without 10^{-6} M RU486 was examined by western blot analysis. Similar results were obtained from three independent experiments. The blots of *Rhob* were quantified by Tanon software and normalized against β -actin. Then the normalized numbers from three independent experiments were compared. The values are expressed as fold of the level of control, as shown in the bar graph of (C) ($X \pm S.D.$, $n = 3$). ** $P < 0.01$ versus control.

the activity of Akt (Leis *et al.* 2004, Chrysis *et al.* 2005, Nuutinen *et al.* 2006), MAPK3/1 and MAPK14 (Miller *et al.* 2005, Qin *et al.* 2005), so we examined the activated status of these kinases in RAW-GR(–) and RAW264.7 cells by western blot. We failed to find a significant difference in phosphorylated MAPK3/1 or MAPK14 level between RAW-GR(–) and RAW-control cells (data not shown), but found about a 79% increase in phosphorylated Akt in RAW-GR(–) cells compared with that of RAW-control cells (Fig. 6A). To validate the effect of Akt on the increased proliferation of RAW-GR(–) cells, we further examined the effect of LY294002, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt pathway, on cell growth by

Figure 4 RNA interference target *Rhob* gene knocks down *Rhob* expression in RAW264.7 cells. Twenty-four hours after transfection, RAW264.7 cells with negative control vector, pSilencer 2.1-U6-*Rhobi*(1), or pSilencer 2.1-U6-*Rhobi*(2), protein expression of RHOB in the cells was assayed by western blot analysis. Similar results were obtained from three independent experiments. The blots of RHOB in these two cells were quantified by Tanon software and normalized against β-actin. The normalized numbers from three independent experiments were compared. The values are expressed as the percentage of the level of RAW-control, as shown in the bar graph ($\bar{X} \pm \text{s.d.}$, $n=3$). ** $P<0.01$ versus RAW264.7 cells transfected with negative control vector.

MTT assay. As shown in Fig. 6B, treatment of cells with 25 μM LY294002 resulted in a more obvious growth repression in RAW-GR(–) cells than that in RAW-control cells, though the effect could be seen in both the two cells. These results indicated that enhanced activity of Akt, but not MAPK3/1 and MAPK14, was also associated with the increased proliferation of RAW-GR(–) cells.

Blocking PI3K/Akt pathway in RAW-GR(–) and RAW264.7 cells by LY294002 and inhibiting Akt activity in RAW264.7 cells by Dex-increased *Rhob* expression of the cells

Given that both PI3K/Akt and *Rhob* are involved in the growth of RAW-GR(–) cells, the relevance between the two signaling pathways was then investigated. RAW-GR(–) cells and its parental RAW264.7 cells were treated with 0–50 μM LY294002 for 12 h, and the expression of *Rhob* mRNA was then detected by real-time PCR. As shown in Fig. 7A, the expression of *Rhob* mRNA in RAW-GR(–) and RAW264.7 cells increased gradually with the dosage of LY294002, and the mRNA level of *Rhob* in RAW-GR(–) cells is about 2.3–

Figure 5 Increase in RHOB protein level or activity inhibits the proliferation of RAW264.7 cells, while decrease in RHOB protein level or activity promotes the cell proliferation. The level or activity of *Rhob* was increased by transfection of RAW264.7 cells with *Rhob*-wt (wild-type *Rhob* cDNA) or *Rhob*-V14 (constitutively active *Rhob* mutant), while the level or activity of *Rhob* was decreased by transfection with *Rhob*-RNAi (*Rhob* RNA interference plasmid) or *Rhob*-N19 (dominant negative mutant *Rhob* plasmid). Twenty-four hours after transfection, the cells were seeded and cultured in duplicate for 2 days in 6-well plate (2×10^5 cells/well) for (A) cytometry or (B) 24-well plate (3×10^4 cells/well) for MTT assay ($\bar{X} \pm \text{s.d.}$). * $P<0.05$ versus cells transfected with blank vector ($n=3$).

and 3.2-fold of the control at 25 and 50 μM LY294002 respectively. The expression of RHOB protein was also further examined. The cells were treated with or without 50 μM LY294002, and the protein level was analyzed by western blot. Figure 7B shows that *Rhob* protein in RAW-GR(–) and RAW264.7 cells increased remarkably when compared with that of control when 50 μM LY294002 was added. These results demonstrated that PI3K/Akt signal was in the upstream of *Rhob* in these two cells and down-regulation of *Rhob* in RAW-GR(–) cells was resulted from the activation of PI3K/Akt pathway.

As shown in Fig. 3, we demonstrated that Dex increased the expression of *Rhob* in RAW264.7 cells at mRNA and protein levels. Does Dex also affect PI3K/Akt activity in RAW264.7 cells. Was the role of Dex on *Rhob* in the cells associated with Akt pathway? Therefore, we detected the effect of Dex on the protein level of p-Akt and *Rhob* in

Figure 6 Enhanced expression of phosphorylated Akt is associated with increased proliferation of RAW-GR(-) cells. (A) Phosphorylated Akt (p-Akt) in RAW-GR(-) and RAW-control cells was detected by western blot analysis. These blots are representative of three independent experiments. Using Tanon software, these blots of p-Akt in RAW-GR(-) and RAW-control cells were quantified and normalized against β-actin. Then the normalized numbers from three independent experiments were compared. The values are expressed as fold of the level of RAW-control, as shown in the bar graph of (A) ($X \pm s.d.$, $n=3$). ** $P < 0.01$ versus RAW-control. RAW-GR(-) and RAW-control cells were seeded at 3×10^4 cells per well onto a 24-well plate in duplicate and cultured in the medium with or without 25 μM LY294002, a specific inhibitor of PI3K/Akt pathway, for 4 days. (B) Proliferation of the cells was monitored by MTT assay ($X \pm s.d.$, $n=3$). * $P < 0.05$ versus control cells without LY294002 treatment.

RAW264.7 cells at the same time. A significant decrease in p-Akt protein expression was observed, following incubation of RAW264.7 cells with 10^{-7} M Dex for 4 h accompanied by an increase in *Rhob* and RU486, which could partially attenuate the down-regulation of p-Akt by Dex (Fig. 7C), indicating that activation of GR is required for p-Akt reduction. These results demonstrated that Dex inhibited the activity of Akt in RAW264.7 cells and the decrease in Akt activity might be resulted in the increase in *Rhob* expression of the cells.

Discussion

In our previous work, we found that the inhibition of GR expression in murine macrophage RAW-GR(-) cells by RNA interference resulted in significant enhancement of cell

Figure 7 Inhibition of Akt activity by LY294002 or Dex up-regulates *Rhob* expression. RAW264.7 and RAW-GR(-) cells were treated with LY294002 as indicated, and the expression of *Rhob* in (A) mRNA and (B) protein levels were detected by real-time PCR and western blot analysis respectively. Similar results were obtained from three independent experiments. * $P < 0.01$ versus 0 μM LY294002. (C) The protein level of p-Akt and RHOB in RAW264.7 cells treated with 10^{-7} M Dex with or without 10^{-6} M RU486 by western blot analysis. Similar results were obtained from three independent experiments.

proliferation (Zhu *et al.* 2004). These results indicated that GR plays an important antiproliferative role in these macrophage cells. Although it is well known that GR mediates GC action by regulating the expression of target genes (Dondi *et al.* 2001, Zhu *et al.* 2004, Bladh *et al.* 2005), but a few target genes have been known to mediate the antiproliferative effect of GC until now.

Our other previous work found that *Rhob* could be induced by GC/GR and it at least partially mediated the antiproliferative effect of GC/GR in ovarian cancer HO-8910 cell lines (Chen *et al.* 2006). In this study, we found that GC also induced the expression of *Rhob* at both mRNA and protein levels in RAW264.7 cells and activation of GR is required for *Rhob* induction. The levels of *Rhob* at both mRNA and protein markedly decreased in RAW-GR(-) and RAW264.7 cells transiently transfected with GR RNA interference vector. Ectopic expression or constructive activation of *Rhob* by transfection with *Rhob*-wt or *Rhob*-V14 obviously inhibited the proliferation of RAW-GR(-) cell. Furthermore,

inhibition of the expression or activity of *Rhob* by transfection with *Rhob*-RNAi or *Rhob*-N19 resulted in a significant increase in cell proliferation of parental RAW264.7 cells, while increase in the expression or activity of *Rhob* by transfection with *Rhob*-wt or *Rhob*-V14 inhibited the proliferation. These results demonstrated that *Rhob* negatively regulated the proliferation of RAW264.7 cells and down-regulation of *Rhob* expression was associated with increased proliferation in RAW-GR(−) cell. It needs to be mentioned that *Rhob* expression and the proliferation of RAW-GR(−) cells showed no significant difference between culture in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 10% charcoal-stripped serum (deprived of GC). The data indicated that the presence or absence of GC had no effect on *Rhob* expression when knock down of GR in RAW-GR(−) cells, while GC promoted the expression of *Rhob* remarkably in RAW264.7 cells through GR mediation. Therefore, we speculated that *Rhob* expression might be mainly regulated by GR in an agonist-independent way.

How GR up-regulates the expression of *Rhob* remains unknown. It is well known that GR up-regulates gene transcription usually through direct binding to GRE in the promoter region of target genes, where it serves to recruit other proteins, such as coactivators involved in transcription (Zhu *et al.* 2004, Bladh *et al.* 2005). However, our previous work demonstrated that the activity of a luciferase reporter gene containing a ~1.9 kb (−1756/+111) fragment of the promoter sequence of human *Rhob* gene was not induced by GC/GR in ovarian cancer HO-8910 cells, indicating that there was no functional GRE in this promoter region (−1765/+111; Chen *et al.* 2006). We also analyzed a 5 kb fragment of the promoter sequence upstream of the transcriptional start site of the *Rhob* gene, and found no consensus GRE (Chen *et al.* 2006). In recent years, evidence has emerged that besides its role in directly regulating gene expression, GR could modulate the activity of several cytoplasmic protein kinases, such as Akt (Leis *et al.* 2004, Chrysis *et al.* 2005, Nuutinen *et al.* 2006), MAPK3/1, and MAPK14 (Miller *et al.* 2005, Qin *et al.* 2005), to play its important roles in regulating cell proliferation, differentiation, and apoptosis. Leis *et al.* (2004) reported that GR inhibited PI3K and Akt activity, which constituted a major mechanism underlying the antitumor effect of GC in skin. Reports also showed that GC/GR induces apoptosis in proliferative chondrocytes and a human follicular lymphoma cell line through suppression of the Akt-PI3K signaling pathway (Chrysis *et al.* 2005, Nuutinen *et al.* 2006). In this study, we also found that except for the down-regulation of *Rhob*, the activity of Akt was enhanced in RAW-GR(−) cells. Furthermore, Dex decreased the expression of p-Akt accompanied with an increase in *Rhob*. Therefore, it is interesting to know whether the increased proliferation of RAW-GR(−) cells is related to the down-regulation of *Rhob* resulted from activation of Akt. Treatment of RAW-GR(−) and RAW-control cells with 25 μM LY294002, a specific inhibitor of PI3K/Akt pathway, resulted in a more obvious growth repression and a more obvious up-regulation of *Rhob* expression

in RAW-GR(−) cells than that in RAW-control cells, though the effect could be seen in both the two cells. These results indicated that inhibition of GR expression in RAW-GR(−) cells leads to activation of Akt, and the latter subsequently down-regulates the expression of *Rhob*, which results in the faster growth of the cells. Jiang *et al.* (2004a,b) also reported that activated Akt by EGFR-Ras signaling suppresses *Rhob* promoter activity by a PI3K/Akt-dependent mechanism.

It has been reported that Akt regulated cell growth progress by controlling the expression and activity of multiple cell cycle regulatory proteins, such as cyclin D1, cyclin E, CDK2, CDK4, and cyclin-dependent kinase inhibitors CDKN1B and CDKN1A (Shukla & Gupta 2007, Lee *et al.* 2008, Seo *et al.* 2008). Wu *et al.* (2008) reported that PRKCA, a regulator of cell proliferation, was highly expressed in the poorly differentiated hepatocellular carcinoma cell lines and the reduction in PRKCA expression resulted in a decrease in the growth rate and the level of cyclin D1 in these cells. In our previous study, we demonstrated that increased proliferation of RAW-GR(−) cells was associated with an increase in PRKCA and decrease in CDKN1B (Zhu *et al.* 2004) and with an increase in CDK2, cyclin D1, and cyclin B1 (unpublished). These results suggest that the role of GR on cell cycle regulatory proteins may be through regulating the expression of Akt and PRKCA in RAW264.7 cells, but it needs further study.

In summary, our experiments demonstrate that *Rhob* plays an important role in the antiproliferative effect of GR on RAW264.7 cells by GR → Akt → *Rhob* pathway and *Rhob* negatively regulated the proliferation of RAW264.7 cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Adnane J, Muro-Cacho C, Mathews L, Sebt SM & Munoz-Antonia T 2002 Suppression of rho B expression in invasive carcinoma from head and neck cancer patients. *Clinical Cancer Research* **8** 2225–2232.
- Bladh LG, Lidén J, Pazirandeh A, Rafter I, Dahlman-Wright K, Nilsson S & Okret S 2005 Identification of target genes involved in the antiproliferative effect of glucocorticoids reveals a role for nuclear factor-(κ)B repression. *Molecular Endocrinology* **19** 632–643.
- Brummer E, Choi JH & Stevens DA 2005 Interaction between conidia, lung macrophages, immunosuppressants, proinflammatory cytokines and transcriptional regulation. *Medical Mycology* **43** S177–S179.

- Chen YX, Li ZB, Diao F, Cao DM, Fu CC & Lu J 2006 Up-regulation of RhoB by glucocorticoids and its effects on the cell proliferation and NF- κ B transcriptional activity. *Journal of Steroid Biochemistry and Molecular Biology* **101** 179–187.
- Chrysis D, Zaman F, Chagin AS, Takigawa M & Savendahl L 2005 Dexamethasone induces apoptosis in proliferative chondrocytes through activation of caspases and suppression of the Akt-phosphatidylinositol 3'-kinase signaling pathway. *Endocrinology* **146** 1391–1397.
- Distelhorst CW 2002 Recent insights into the mechanism of glucocorticoid-induced apoptosis. *Cell Death and Differentiation* **9** 6–19.
- Dondi D, Maggi R, Scaccianoce E, Martini L, Motta M & Poletti A 2001 Expression and role of functional glucocorticoid receptors in the human androgen-independent prostate cancer cell line, DU145. *Journal of Molecular Endocrinology* **26** 185–191.
- Forget MA, Desrosiers RR, Del M, Moumdjian R, Shedid D, Berthelet F & Beliveau R 2002 The expression of rho proteins decreases with human brain tumor progression: potential tumor markers. *Clinical and Experimental Metastasis* **19** 9–15.
- Frankfurt O & Rosen ST 2004 Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates. *Current Opinion in Oncology* **16** 553–563.
- Heitzer MD, Wolf IM, Sanchez ER, Witchel SF & DeFranco DB 2007 Glucocorticoid receptor physiology. *Reviews in Endocrine and Metabolic Disorders* **8** 321–330.
- Huang M & Prendergast GC 2006 RhoB in cancer suppression. *Histology and Histopathology* **21** 213–218.
- Jiang K, Delarue FL & Sebtì SM 2004a EGFR, ErbB2 and Ras but not Src suppress RhoB expression while ectopic expression of RhoB antagonizes oncogene-mediated transformation. *Oncogene* **23** 1136–1145.
- Jiang K, Sun J, Cheng J, Djeu JY, Wei S & Sebtì S 2004b Akt mediates Ras downregulation of RhoB, a suppressor of transformation, invasion, and metastasis. *Molecular and Cellular Biology* **24** 5565–5576.
- Kudawara I, Ueda T, Yoshikawa H, Miyama T, Yamamoto T & Nishizawa Y 2001 *In vivo* inhibition of tumour growth by dexamethasone in murine osteosarcomas. *European Journal of Cancer* **37** 1703–1708.
- Lee JY, Jang KS, Shin DH, Oh MY, Kim HJ, Kim Y & Kong G 2008 Mel-18 negatively regulates INK4a/ARF-independent cell cycle progression via Akt inactivation in breast cancer. *Cancer Research* **68** 4201–4209.
- Leis H, Page A, Ramirez A, Bravo A, Segrelles C, Paramio J, Barettono D, Jorcano JL & Perez P 2004 Glucocorticoid receptor counteracts tumorigenic activity of Akt in skin through interference with the phosphatidylinositol 3-kinase signaling pathway. *Molecular Endocrinology* **18** 303–311.
- Liu AX, Rane N, Liu JP & Prendergast GC 2001 RhoB is dispensable for mouse development, but it modifies susceptibility to tumor formation as well as cell adhesion and growth factor signaling in transformed cells. *Molecular and Cellular Biology* **21** 6906–6912.
- Lloberas J, Soler C & Celada A 1998 Dexamethasone enhances macrophage colony stimulating factor- and granulocyte macrophage colony stimulating factor-stimulated proliferation of bone marrow-derived macrophages. *International Immunology* **10** 593–599.
- Mazieres J, Antonia T, Daste G, Muro-Cacho C, Berchery D, Tillement V, Pradines A, Sebtì S & Favre G 2004 Loss of RhoB expression in human lung cancer progression. *Clinical Cancer Research* **10** 2742–2750.
- Miller AL, Webb MS, Copik AJ, Wang Y, Johnson BH, Kumar R & Thompson EB 2005 p38 Mitogen-activated protein kinase (MAPK) is a key mediator in glucocorticoid-induced apoptosis of lymphoid cells: correlation between p38 MAPK activation and site-specific phosphorylation of the human glucocorticoid receptor at serine 211. *Molecular Endocrinology* **19** 1569–1583.
- Nuutinen U, Postila V, Matto M, Eeva J, Ropponen A, Eray M, Riikonen P & Pelkonen J 2006 Inhibition of PI3-kinase-Akt pathway enhances dexamethasone-induced apoptosis in a human follicular lymphoma cell line. *Experimental Cell Research* **312** 322–330.
- Pagniello KB, Bols NC & Lee LE 2002 Effect of corticosteroids on viability and proliferation of the rainbow trout monocyte/macrophage cell line, RTS11. *Fish and Shellfish Immunology* **13** 199–214.
- Prendergast GC 2001 Actin' up: RhoB in cancer and apoptosis. *Nature Reviews. Cancer* **1** 162–168.
- Qin JM, Yan HX, Wan XW, Liu SQ, Zeng JZ, Cao HF, Wu MC & Wang HY 2005 Effects of signal regulatory protein alpha1 on proliferation of hepatocellular carcinoma: a preliminary study. *Hepatobiliary and Pancreatic Diseases International* **4** 244–248.
- Sakai M, Biwa T, Matsumura T, Takemura T, Matsuda H, Anami Y, Sasahara T, Kobori S & Shichiri M 1999 Glucocorticoid inhibits oxidized LDL-induced macrophage growth by suppressing the expression of granulocyte/macrophage colony-stimulating factor. *Arteriosclerosis, Thrombosis, and Vascular Biology* **19** 1726–1733.
- Seo JM, Kim TJ, Jin YR, Han HJ, Ryu CK, Sheen YY, Kim DW & Yun YP 2008 YSK2821, a newly synthesized indoleione derivative, inhibits cell proliferation and cell cycle progression via the cell cycle-related proteins by regulating phosphatidylinositol-3 kinase cascade in vascular smooth muscle cells. *European Journal of Pharmacology* **586** 74–81.
- Shukla S & Gupta S 2007 Apigenin-induced cell cycle arrest is mediated by modulation of MAPK, PI3K-Akt, and loss of cyclin D1 associated retinoblastoma dephosphorylation in human prostate cancer cells. *Cell Cycle* **6** 1102–1114.
- Stamatakis K, Cernuda-Morollon E, Hernandez-Perera O & Perez-Sala D 2002 Isoprenylation of RhoB is necessary for its degradation. A novel determinant in the complex regulation of RhoB expression by the mevalonate pathway. *Journal of Biological Chemistry* **277** 49389–49396.
- Wu TT, Hsieh YH, Hsieh YS & Liu JY 2008 Reduction of PKC alpha decreases cell proliferation, migration, and invasion of human malignant hepatocellular carcinoma. *Journal of Cellular Biochemistry* **103** 9–20.
- Yamamoto T, Nishiguchi M, Inoue N, Goto HG, Kudawara I, Ueda T, Yoshikawa H, Tanigaki Y & Nishizawa Y 2002 Inhibition of murine osteosarcoma cell proliferation by glucocorticoid. *Anticancer Research* **22** 4151–4156.
- Zhang J, Zhu J, Bu X, Cushion M, Kinane TB, Avraham H & Koziel H 2005 Cdc42 and RhoB activation are required for mannose receptor-mediated phagocytosis by human alveolar macrophages. *Molecular Biology of the Cell* **16** 824–834.
- Zhu XY, Liu YJ, Lu J & Xu RB 2004 Knockdown of glucocorticoid receptor expression by RNA interference promotes cell proliferation in murine macrophage RAW264.7 cells. *Journal of Steroid Biochemistry and Molecular Biology* **92** 375–382.

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