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Rottlerin Inhibits Migration of Follicular Thyroid Carcinoma Cells by PKCδ-Independent Destabilization of the Focal Adhesion Complex

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ABSTRACT

This study examined the effect of rottlerin on the focal adhesion-mediated cell migration of CGTH W-2 human follicular thyroid carcinoma cells. Rottlerin (10 μM) resulted in decreased adhesion of CGTH W-2 cells to matrix substance, which was correlated with metastatic potential. Rottlerin treatment also resulted in a marked reduction in the migration of CGTH W-2 cells. Protein levels of integrin β1, FAK, and paxillin were decreased by rottlerin. Consistent with this, immunostaining of FAK, vinculin, and paxillin revealed disassembly of the focal adhesions. Disruption of actin stress fibers was noted, which was compatible with reduced expression levels and activities of Rac-1 and Rho. The effect of rottlerin on cell migration was not attributable to inhibition of PKCδ activity since siRNA knockdown of PKCδ did not recapitulate the effects of rottlerin on cell adhesion and migration. Furthermore, activation of PKCδ by phorbol esters failed to restore the rottlerin-inhibited migratory ability. The mitochondrial uncoupler, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone, was able to mimic several rottlerin's effects. In summary, we demonstrated that rottlerin inhibits the migration of CGTH W-2 cells by disassembly of focal adhesion complexes in a PKCδ-independent manner, and might play as a mitochondrial uncoupler role in these events. J. Cell. Biochem. 110: 428–437, 2010.

KEY WORDS: FOCAL ADHESION COMPLEX; Rho; Rac-1; PROTEIN KINASE Cδ; ROTTLERIN; MIGRATION

Thyroid cancer is the most common neoplasm of the endocrine system. The incidence of this disease has been rising steadily during past decades [Davies and Welch, 2006]. The prognosis of follicular thyroid carcinoma, one of the more malignant variants, correlates highly with metastatic status [Crile et al., 1985]. Therefore, understanding mechanisms of follicular thyroid cancers metastasis may facilitate the advancement of clinical management.

Cell migration in metastasis involves turnover of focal adhesion complexes, which act as bridges between the intracellular cytoskeleton and the transmembrane integrins. Integrins anchor cells to the extracellular matrix and relay signals therein. Other than integrins, focal adhesion complex consists of several component proteins, including focal adhesion kinase (FAK), vinculin, and paxillin [Mitra et al., 2005]. Of these, FAK and its activated form, pY397FAK (pFAK), play crucial roles in mediating the assembly and disassembly of the complex [Mitra et al., 2005]. Once activated by clustering of integrins, pFAK relays signaling cascade by activating downstream GTPases RhoA and Rac-1, leading to polymerization of

Abbreviations used: FAK, focal adhesion kinase; DMSO, dimethyl sulfoxide; FCCP, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone; MTT, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; PMA, phorbol myristate acetate; PKCδ, protein kinase Cδ; siRNA, small interfering RNA.

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Chien-Jung Lin and Chieh-Yu Lin contributed equally to this study.

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actin and formation of stress fibers [Carragher and Frame, 2004]. Aberrant expression of focal adhesion components, including integrins, FAK, and Rho GTPases, has been found in invasive thyroid cancers [Demeure et al., 1992; Owens et al., 1996; Schmitz et al., 2000], implying that focal adhesion might play an important role in the malignant phenotype of thyroid cancers.

Protein kinase Cδ (PKCδ), an atypical PKC [Kikkawa et al., 2002], plays a crucial role in the control of apoptosis, cell migration, and cytoskeleton remodeling [Kikkawa et al., 2002; Steinberg, 2004]. These cellular functions are important regulators of tumor progression and metastasis. Studies of PKCδ on different cell types, however, have resulted in conflicting results on whether it is associated with increased or decreased motility [Jackson et al., 2005; Kharait et al., 2006]. Rottlerin, first isolated from *Mallotus philippinensis*, is originally used as a specific PKCδ inhibitor [Soltoff, 2007]. Rottlerin treatment is associated with decreased cellular motility in various cancer models [Kharait et al., 2006]. The effective mechanism by which rottlerin acts, however, has been recently debated [Soltoff, 2007].

In this study, we revealed that rottlerin reduced cell motility and changed the morphology of follicular thyroid carcinoma cells. Concordantly, the adhesion of CGTH W-2 cells to substratum matrix was curtailed by rottlerin. We also showed decreased integrin β1, FAK, and focal adhesion proteins. We found reduced protein level and activity of Rho GTPases, which is accompanied by disrupted stress fiber formation. We exclude the hypothesis that rottlerin exerts its effects via the inhibition of PKCδ and suggest that mitochondrial uncoupling might contribute to the rottlerin's effect. These results indicate an anti-tumor role of rottlerin in follicular thyroid carcinoma and could unveil an important new approach to its treatment.

MATERIALS AND METHODS

CELL CULTURE

The human metastatic follicular thyroid carcinoma cell line CGTH W-2, a generous gift from Dr. Jen-Der Lin, was maintained in growth medium (RPMI 1640 medium containing 10% fetal bovine serum [Gibco, Rockville, MD]), as described previously [Huang et al., 1998].

siRNA KNOCKDOWN OF PKCδ

CGTH W-2 cells in the number of 10^6 were trypsinized and resuspended in 100 μl of Nucleofactor solution (Amaxa, Germany). One hundred micromolars of small interfering RNA (siRNA) duplexes: UUCUGGAAUAUAGUGUCCCGG (Ambion, USA) were electroporated into the cells as advised in the instruction manual. The cells were seeded onto 6-cm plates immediately after transfection. The cells were harvested 48 h after transfection for Western blot studies. For the migration assay, cells were allowed to recover for 48 h after transfection. The cells were then resuspended and used for the migration assay as described [Lin et al., 2006].

ANTIBODIES AND CHEMICALS

The antibodies used in this study were listed in Table I. Rottlerin was from Biomol (Plymouth Meeting, PA). The concentration used was 10 μM throughout the experiment. Phorbol myristate acetate (PMA) and 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were from Sigma.

TABLE I. List of Antibodies Used in This Study

Antigen	Animal	Source
Primary antibodies		
FAK	Mouse	Transduction Labs
Integrin β1	Mouse	Transduction Labs
Vinculin	Mouse	Sigma
GAPDH	Mouse	Abcam
RhoA	Mouse	Upstate
Paxillin	Mouse	Transduction Labs
pY118 paxillin	Mouse	Transduction Labs
PKCδ	Rabbit	Santa Cruz
pT507 PKCδ	Rabbit	Santa Cruz
Rac-1	Rabbit	Cytoskeleton
pY397 FAK	Rabbit	Biosource
Secondary antibodies		
Rabbit IgG FITC-conjugate	Goat	Sigma
Mouse IgG FITC-conjugate	Goat	Sigma
Mouse IgG AP-conjugate	Goat	Promega
Rabbit IgG AP-conjugate		Promega
Phalloidin FITC-conjugate		Sigma

MTT ASSAY

CGTH W-2 cells (2×10^4 cells/well) were plated on 24-well plates and incubated in growth medium with or without rottlerin for 24 h. The cells were then assayed as described previously [Chen et al., 2006].

ASSAY FOR APOPTOSIS AND NECROSIS

CGTH W-2 cells (10^4 cells/slip) were plated on coverslips and incubated in growth medium with or without rottlerin for 24 h. The cells were fixed, stained with 4′,6-diamidino-2-phenylindole dilactate (DAPI, Sigma) or propidium iodide (PI, Sigma) and quantified as described before [Chen et al., 2006]. Two hundred cells were counted in each coverslip. The experiments were done in triplicates.

FLOW CYTOMETRY

CGTH W-2 cells, 1×10^6 cells/dish, were plated in 6 cm dishes. After incubation with growth medium with or without rottlerin for 24 h, the cells were harvested. Fixation and staining of PI was done and flow cytometry was performed as previously described.

IMMUNOFLUORESCENCE MICROSCOPY

CGTH W-2 cells were immunostained and images acquired as described previously [Chen et al., 2006; Lin et al., 2006].

MIGRATION AND WOUND-HEALING ASSAYS

The migration assay was carried out as described previously [Lin et al., 2006]. We seeded 1×10^4 CGTH W-2 cells in the upper chamber of a Transwell apparatus with 8 μm pore size (Costar, Acton, MA). After cell attachment, 0.5 ml of growth medium with rottlerin or DMSO vehicle was added to the lower well. After 20 h, the polycarbonate membranes were fixed in 10% formalin for 10 min and stained with Coomassie Brilliant Blue G250 (Sigma) for 5 min. The number of cells that had migrated to the reverse surface of the membrane was counted in three randomly selected fields under light microscopy. For the wound-healing assay, CGTH W-2

cells were grown to confluence in growth medium, then straight wounds were created using sterile pipette tips and the medium replaced with growth medium containing either rottlerin or DMSO. Photomicrographs were taken at 0 h and designated endpoint. Wound closure was expressed as μm/h.

CELL ADHESION ASSAY

Cell adhesion assays were performed in 24-well plates (Nunc, Naperville, IL) pre-coated with 5 μg/ml fibronectin (Sigma). CGTH-W-2 cells treated with rottlerin or siRNA as indicated were and suspended in growth medium. Cell suspension (1×10^5/ml) was seeded onto the wells and incubated for 1 h at 37°C. The nonadherent cells were removed by washing three times with PBS, and the adherent cells were fixed with 10% formalin. Photomicrographs were taken and the amount of remained cell was counted.

WESTERN BLOT ANALYSIS

Western blotting of equal amounts of whole cell lysates was carried out as described previously [Lin et al., 2006]. To prepare the membrane fraction, the cells were collected from culture dishes and subjected to seven cycles of freeze-thawing in RIPA buffer (20 mM HEPES, pH 7.4, 280 mM sucrose, 50 mM NaCl, 2 mM EDTA, 1 mM PMSF, 2.5 μg/ml of pepstatin A, 2.5 μg/ml of leupeptin, 1 μg/ml of aprotinin, 1 mM NaF, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, and 5 mM β-glycerol phosphate). All subsequent steps were at 4°C. The samples were centrifuged at 500*g* for 10 min to remove nuclei and the supernatant centrifuged at 13,000*g* for 1 h. The resultant pellets were solubilized in NP buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% NP-40, and inhibitor mixture), and taken as the membrane fraction.

Rho GTPase ACTIVITY ASSAYS

CGTH W-2 cells were grown to confluence in 6 cm dishes. After 1 h incubation in rottlerin or DMSO, the cells were washed with PBS and suspended in the lysis buffer provided with the RhoA and Rac1 Activation Assay Biochem Kits (Cytoskeleton, Denver, CO). The lysates were processed and assayed according to the manufacturer's instructions.

STATISTICAL ANALYSIS

All results were analyzed using Student's *t*-test, with a *P* value of less than 0.05 being considered significant. The results are expressed as the mean ± SD. The results of the Western analysis were normalized to those for the GAPDH or β-actin control.

RESULTS

ROTTLERIN ALTERS THE MORPHOLOGY OF CGTH W-2 CELLS

We first examined the morphology of CGTH W-2 cells treated with 10 μM rottlerin or 0.1% DMSO solvent for 24 h. In the presence of DMSO, CGTH W-2 cells retained a polygonal cell shape, similar to those grown in control medium (Fig. 1A). However, we observed striking morphological changes in the rottlerin-treated group. Retraction of membrane ruffles was seen, giving the cells a shrunken appearance (Fig. 1A). We therefore proposed that rottlerin-treated cells have impaired cytoskeleton structural integrity and/or decreased cell-substratum anchoring.

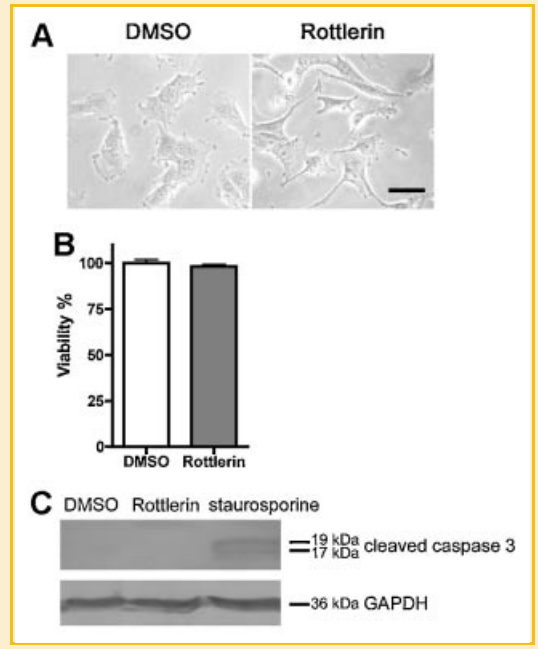

Fig. 1. Effects of rottlerin on CGTH W-2 cell morphology and survival. A: Phase photomicrographs of CGTH W-2 cells cultured for 24 h in growth medium with 10 μM rottlerin or 0.1% DMSO solvent. Note the rottlerin-treated cells have more slender cell processes. Bar = 10 μm. B: Cell survival. Cell number was counted after 24 h incubation with 10 μM rottlerin or 0.1% DMSO solvent (n = 3). C: Western blot analysis of (active) cleaved caspase-3 in cells treated with rottlerin or DMSO for 24 h. Staurosporine is known to induce apoptosis and serves as a positive control for cleaved caspase 3.

ROTTLERIN INHIBITS CELLULAR ADHERENCE AND MIGRATION OF CGTH W-2 CELLS

The morphological change in rottlerin-treated cells suggested decreased cell-substratum anchoring. The degree of which cancer cells adhere to substratum is correlated to their malignancy or metastatic activity [Zhong et al., 2005]. To assess the effect of rottlerin on cell adhesion to ECM proteins, we performed cell adhesion assay. As shown in Figure 2A, rottlerin treatment for 24 h reduced the number of adherent cells on the Matrigel-coated substratum to 39% of the control.

To determine whether this effect changed cellular mobility, we performed migration and wound-healing assays, both of which are widely used to characterize the migratory property of tumor cells. In migration assay, 20 h of incubation with rottlerin reduced the number of migrating cells to about 36% compared to the control group (Fig. 2B). In addition, the wound-healing assay revealed the cells cultured with rottlerin for 20 h closed the cleft 89% slower than DMSO-treated ones (Fig. 4B).

Since the inhibitory effects on cell adhesion and migration could possibly be an artifact caused by rottlerin-induced cell death, we counted the numbers of adherent cells to assess cell survival. We found that 24 h of rottlerin incubation did not result in a significant difference in cell number (Fig. 1B). This finding is further confirmed

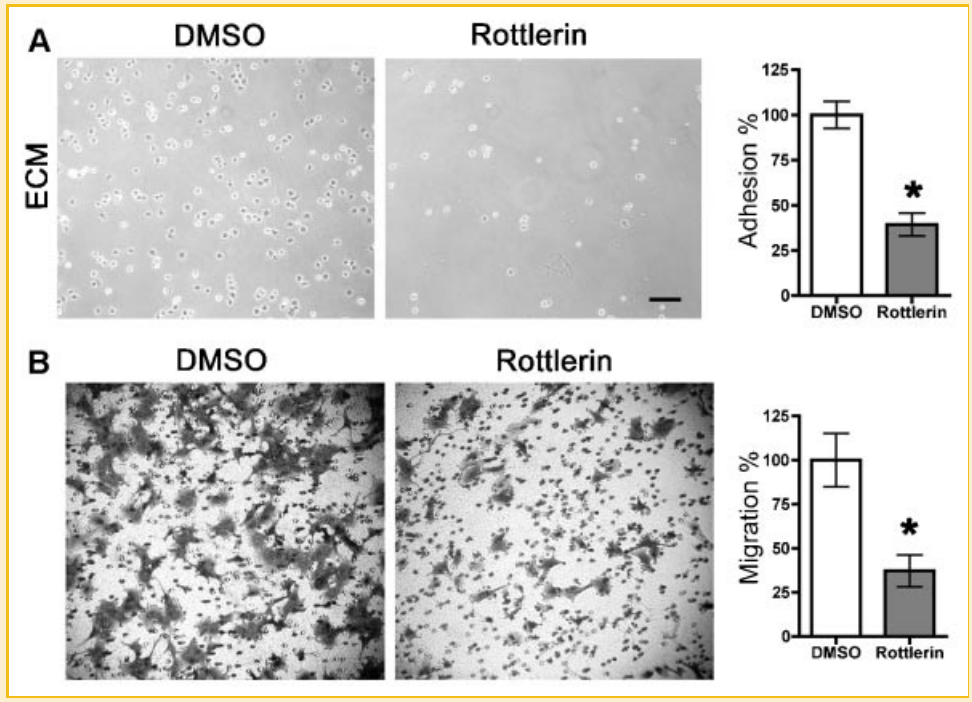

Fig. 2. Effects of rottlerin on the adhesive and migratory behavior of CGTH W-2 cells. A: Adhesion assay. Suspended cells were seeded onto wells coated with Matrigel. After 1 h incubation the nonadherent cells were removed and the adherent cells fixed and counted. Bar = 100 μm (n = 3). B: Migration assay. After 20 h incubation with 0.1% DMSO or 10 μM rottlerin, the number of CGTH W-2 cells crossing 8 μm pores was counted (n = 3).

by MTT cell survival assay (data not shown). We also performed PI vital stain, fluorescent nuclear morphometric analysis, flow cytometry (data not shown), and caspase-3 immunoblotting (Fig. 1C) to quantify the extent of apoptosis. Staurosporin treatment serves as a positive control for inducing cell apoptosis and this group shows positive reaction for cleaved caspase 3 (17 and 19 kDa) (Fig. 1C). Rottlerin treatment did not induce necrosis or apoptosis.

Thus, the decrease in migration could not be attributed to a difference in cell number or viability. Together, these observations clearly demonstrate that rottlerin treatment reduced the adhesion and migratory ability of CGTH W-2 cells.

ROTTLERIN REDUCES THE DISTRIBUTION OF FOCAL ADHESION PLAQUES AND THE EXPRESSION OF FOCAL ADHESION PROTEINS IN CGTH W-2 CELLS

To determine whether the integrin/focal adhesion signaling mediated rottlerin-induced changes in CGTH W-2 cells, we first looked at the expression of integrin β1 by Western blotting. The protein level of integrin β1 was reduced after rottlerin treatment for 24 h (Fig. 3B). We then examined the expression of focal adhesion components by immunostaining and Western blot analysis. In mock-treated cells, staining for paxillin and its activated form, phospho-paxillin (p-paxillin), was mostly found along the cell border, with some staining under the cell body (Fig. 3A,a,c). We observed decreased staining for paxillin along the cell border in rottlerin-treated cells (Fig. 3A,b); staining for p-paxillin was hardly detectable (Fig. 3A,d). Another focal adhesion protein, vinculin, exhibited similar staining pattern (Fig. 3A,i,j). This indicated the structure of focal adhesions was perturbed after 24 h incubation with rottlerin. Indeed, immunostaining for FAK and pFAK localized to focal adhesions in the DMSO-treated control cells (Fig. 3A,e,g), whereas staining for both molecules was largely absent in the rottlerin-treated cells (Fig. 3A,f,h). In accordance with these staining results, protein levels of paxillin, p-paxillin, FAK, and pFAK were reduced in cells treated with rottlerin for 24 h (Fig. 3B). Taken together, these studies support that rottlerin exerts its anti-migratory effects by disassembly of the focal adhesion complex via the integrin/focal adhesion pathway.

ROTTLERIN DISRUPTS STRESS FIBERS AND REDUCES THE EXPRESSION AND ACTIVITY OF Rac-1 AND RhoA

Given that activated FAK relay upstream signal to Rho GTPases, which subsequently regulate cytoskeleton reorganization, we then asked whether this process might contribute to the machinery of rottlerin-induced migration arrest. We first examined the distribution of stress fibers, the formation of which is associated with an increase in cell motility [Schmitz et al., 2000; Mitra et al., 2005]. By FITC-phalloidin staining, we found that stress fibers in mock-treated cells were intact, as shown by the multiple long fibers within the cytoplasm (Fig. 3C,a). In contrast, in cells treated with rottlerin for 24 h, the stress fibers were disrupted and appeared as residual short fibrils and dots within the cell (Fig. 3C,b). We then examined the protein level and activity of RhoA and Rac-1. Western blots showed that the protein level of total RhoA and Rac-1 was reduced after 24 h incubation with rottlerin (Fig. 3D). Likewise, the activity of RhoA and Rac-1, as determined by GTPase pull-down assays, was found decreased after 24 h of rottlerin treatment (Fig. 3E). These findings suggested that stress fiber depolymerization caused by reduced

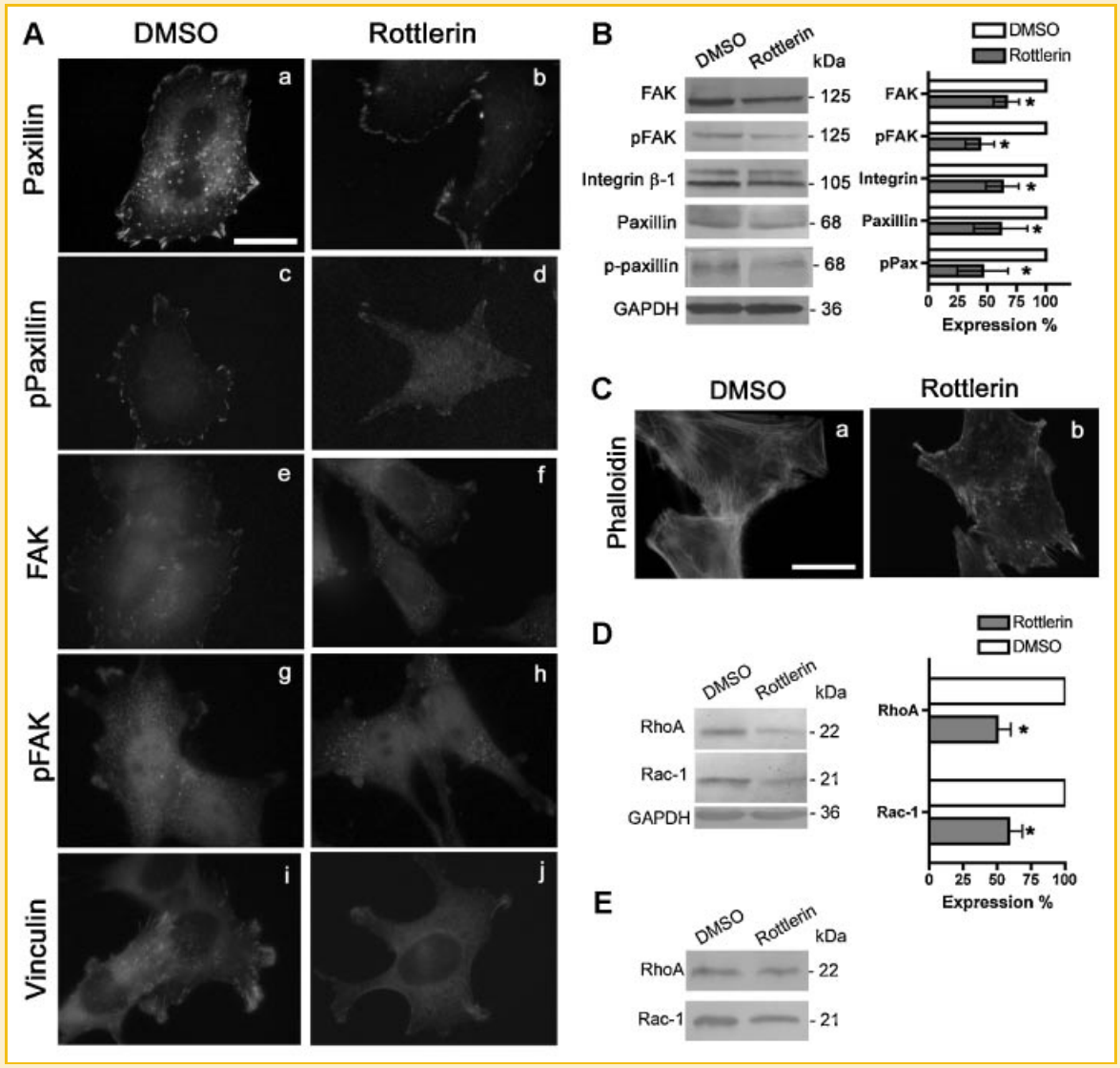

Fig. 3. Effects of rottlerin on the expression of focal adhesion proteins, Rho GTPases, and stress fibers in CGTH W-2 cells. A: Immunofluorescence staining for focal adhesion proteins. CGTH W-2 cells were cultured in 10 μM rottlerin or 0.1% DMSO for 24 h, and then immunostained for focal adhesion proteins. Bar = 10 μm. B: Western blot analysis of focal adhesion proteins in CGTH W-2 cells treated with DMSO or 10 μM rottlerin for 24 h. GAPDH was used as the loading control (n = 6). C: Stress fiber disruption after rottlerin treatment. Cells were treated with 0.1% DMSO or 10 μM rottlerin for 24 h, and stained with FITC-phalloidin. Bar = 10 μm. D: Decreased expression of RhoA and Rac-1 after rottlerin treatment. Cells treated with 0.1% DMSO or 10 μM rottlerin for 24 h were analyzed for total RhoA and Rac1 by Western blotting. GAPDH was used as the loading control (n = 6); $^{*}P < 0.05$. E: Decreased activity of RhoA and Rac-1 after rottlerin treatment. Cells incubated with DMSO or rottlerin for 24 h were subjected to RhoA and Rac-1 pull-down assays (n = 2).

RhoA and Rac-1 activity is the downstream effector of migration inhibition after rottlerin treatment.

INHIBITION OF MIGRATION BY ROTTLERIN IS INDEPENDENT OF PKC

Multiple lines of evidence suggest the ineffectiveness of rottlerin as a PKCδ inhibitor [Soltoff, 2007]. We therefore addressed whether the migration-inhibitory effect of rottlerin on CGTH W-2 cells was due to PKCδ inhibition.

We first tested whether rottlerin suppressed PKCδ activity in CGTH W-2 cells. Since activated PKCδ translocates to the cell membrane [Kikkawa et al., 2002], we prepared membrane fractions from the rottlerin and/or PMA-treated cells described below and assayed the relative abundance of active membrane-bound phospho-PKCδ (pPKCδ). CGTH W-2 cells were incubated with rottlerin or solvent vehicle for 0, 0.5, or 1 h before co-treatment with 200 nM PMA, a well-known activator of PKC [Koike et al., 2006]. All groups were harvested 1 h after PMA addition. Cells treated with PMA alone displayed a striking increase in membrane-bound pPKCδ level (Fig. 4A). The increase in membrane-bound PKCδ was not abolished by rottlerin pretreatment (30 min and 1 h). Also, the levels of membranous pPKCδ in cells incubated with rottlerin alone did not differ significantly from that of DMSO-treated cells (Fig. 4A). These results confirmed that rottlerin failed to suppress PKCδ activity in CGTH W-2 cells. Moreover, in wound-healing assay, cells co-treated

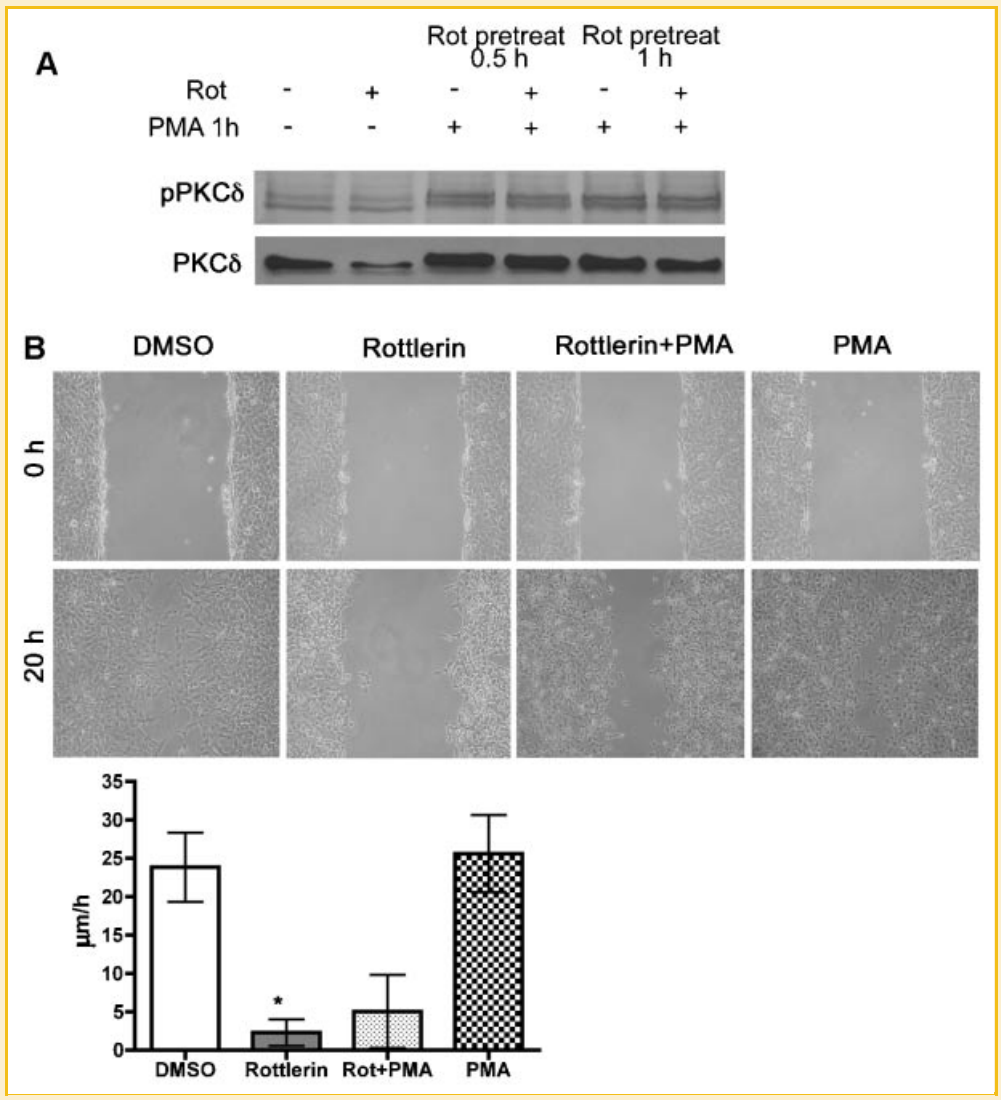

Fig. 4. The effects of rottlerin are independent of PKCδ. A: Protein levels of phospho-PKCδ and PKCδ in the membrane fractions of CGTH W-2 cells pretreated with 10 μM rottlerin for indicated period (30 min, 60 min) and harvested after treatment with PMA or DMSO for 1 h (n = 2). B: Rottlerin treatment inhibits wound closure in PMA-treated cells. The difference between the DMSO and rottlerin groups was statistically significant (n = 6); $^{*}P < 0.05$.

with rottlerin and PMA for 20 h exhibited reduced migration behavior, similar to that of rottlerin-treated cells (Fig. 4B). This suggested that rottlerin inhibited migration of CGTH W-2 cells in a PKCδ-independent manner.

We then tested whether PKCδ downregulation using siRNA recapitulates rottlerin-induced effects. CGTH W-2 cells were resuspended and electroporesed with siRNA to PKCδ. Total cell lysates were harvested 48 h after transfection and analyzed by electrophoresis to demonstrate knockdown efficiency. Thyroid cells expressed elevated levels of α, β, δ, and ε as quantified by Western blot analyses [Green et al., 1997]. siRNA PKCδ significantly suppressed the expression of PKCδ by 65%, and did not affect the expression levels of PKCα, β1, ε, and μ (Fig. 5A), indicating the specificity of this siRNA. In siNeg/PMA groups, PMA increased PKCδ phosphorylation to 165%, but after siRNA to PKCδ, PMA did not significantly induce the phosphorylation of the remaining PKCδ (Fig. 5C). Interestingly, the morphology of cells subjected to PKCδ siRNA transfection was similar to that of control cells (Fig. 5C). Transfection with siRNA to PKCδ did not reduce the number of adherent cells in the adhesion assay (Fig. 5D). Likewise, introduction of PKCδ siRNA did not alter the migratory properties of CGTH W-2 cells (Fig. 5E). In addition, Western blot analysis revealed that downregulation of PKCδ did not result in decreased levels of focal adhesion complex proteins (Fig. 5F). Taken together, we clearly excluded the possibility that rottlerin exerts its inhibitory effects by inhibiting PKCδ.

MITOCHONDRIAL UNCOUPLING RECAPITULATES ANTI-MIGRATORY EFFECTS OF ROTTLERIN

Rottlerin has been proposed to function as a mitochondrial uncoupler [Soltoff, 2001]. To examine whether mitochondrial uncoupling recapitulates the inhibitory effects of rottlerin, we

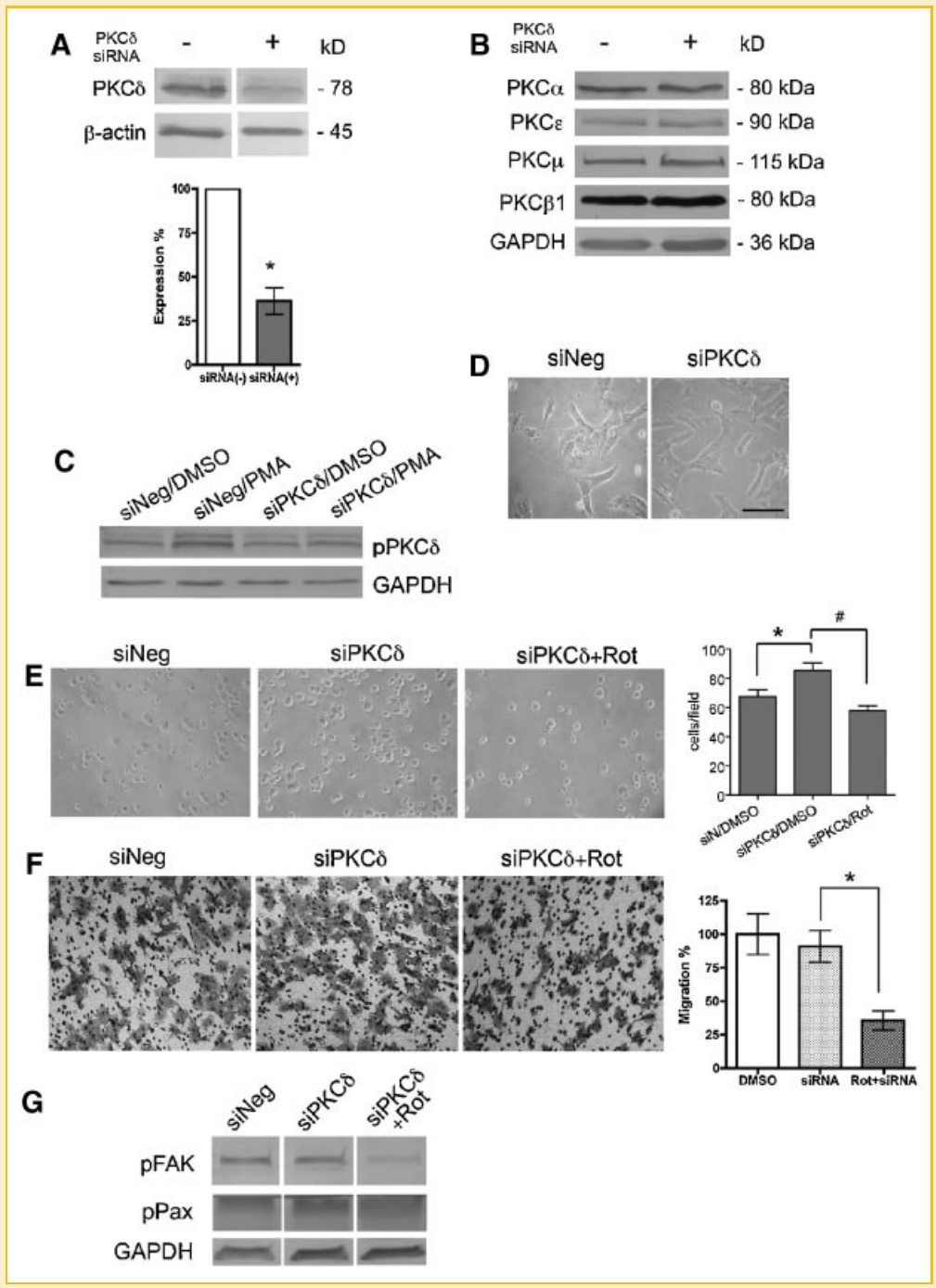

Fig. 5. siRNA knockdown of PKCδ does not decrease CGTH W-2 cell migration. A: Protein levels of PKCδ in CGTH W-2 cells electroporesed with siRNA targeting at PKCδ for 48 h. Densitometric analysis of PKCδ expression normalized with β-actin signal is shown in the bar graph ($n = 3$); $^{*}P < 0.05$. B: Protein levels of several PKC isoforms in CGTH W-2 cells electroporesed with siRNA targeting at PKCδ for 48 h. GAPDH is loading control. C: Protein levels of phosphorylated PKCδ in CGTH W-2 cells electroporesed with siRNA PKCδ for 48 h and activated by PMA. D: Phase photomicrographs of CGTH W-2 cells electroporesed with siRNA targeting at PKCδ for 48 h. Note the siRNA-treated cells have similar morphology to siNeg cells. Bar = 10 μm. E: Adhesion assay. Cells electroporesed with PKCδ siRNA for 48 h were resuspended and seeded onto 24-well plates coated with fibronectin. After 1 h incubation, the nonadherent cells were removed and the remaining adherent cells counted ($n = 3$); $^{*,\#}P < 0.05$. F: Migration assay. Cells electroporesed with PKCδ siRNA for 48 h were resuspended and seeded onto Transwell apparatuses. After 20 h incubation with 0.1% DMSO or 10 μM rottlerin, the number of CGTH W-2 cells crossing 8 μm pores was counted. Note the difference between siNeg/DMSO and siPKCδ/DMSO groups were not statistically significant, whereas that of siPKCδ and siPKCδ + rottlerin groups was statistically significant ($n = 3$). G: Western blot analysis of focal adhesion proteins in CGTH W-2 cells treated with DMSO or siRNA targeting at PKCδ. GAPDH was used as the loading control ($n = 2$).

treated CGTH W-2 cells with 10 μM carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), which at this concentration is a well-established mitochondria uncoupler. We found that FCCP-treated CGTH W-2 cells exhibited behaviors comparable to those of rottlerin-treated cells, including alteration of morphology (Fig. 6A), decreased wound-healing property (Fig. 6C), and decreased levels of focal adhesion proteins (Fig. 6D). Although these findings do not establish a causal relationship, they are consistent with the notion that rottlerin's inhibitory effects might be due to mitochondrial uncoupling characteristics.

DISCUSSION

In this study, we investigated the effects of rottlerin on the migratory behavior of CGTH W-2 follicular thyroid carcinoma cells. The cells treated with rottlerin presented with altered morphology, reduced adhesion to extracellular matrix, and decreased migration ability. We found decreased protein levels of integrin β1, FAK, focal adhesion complex constituents, and reduced activity of Rho GTPases. We also observed depolymeriztion of stress fibers. These findings support an integrin/focal adhesion/cytoskeleton signaling

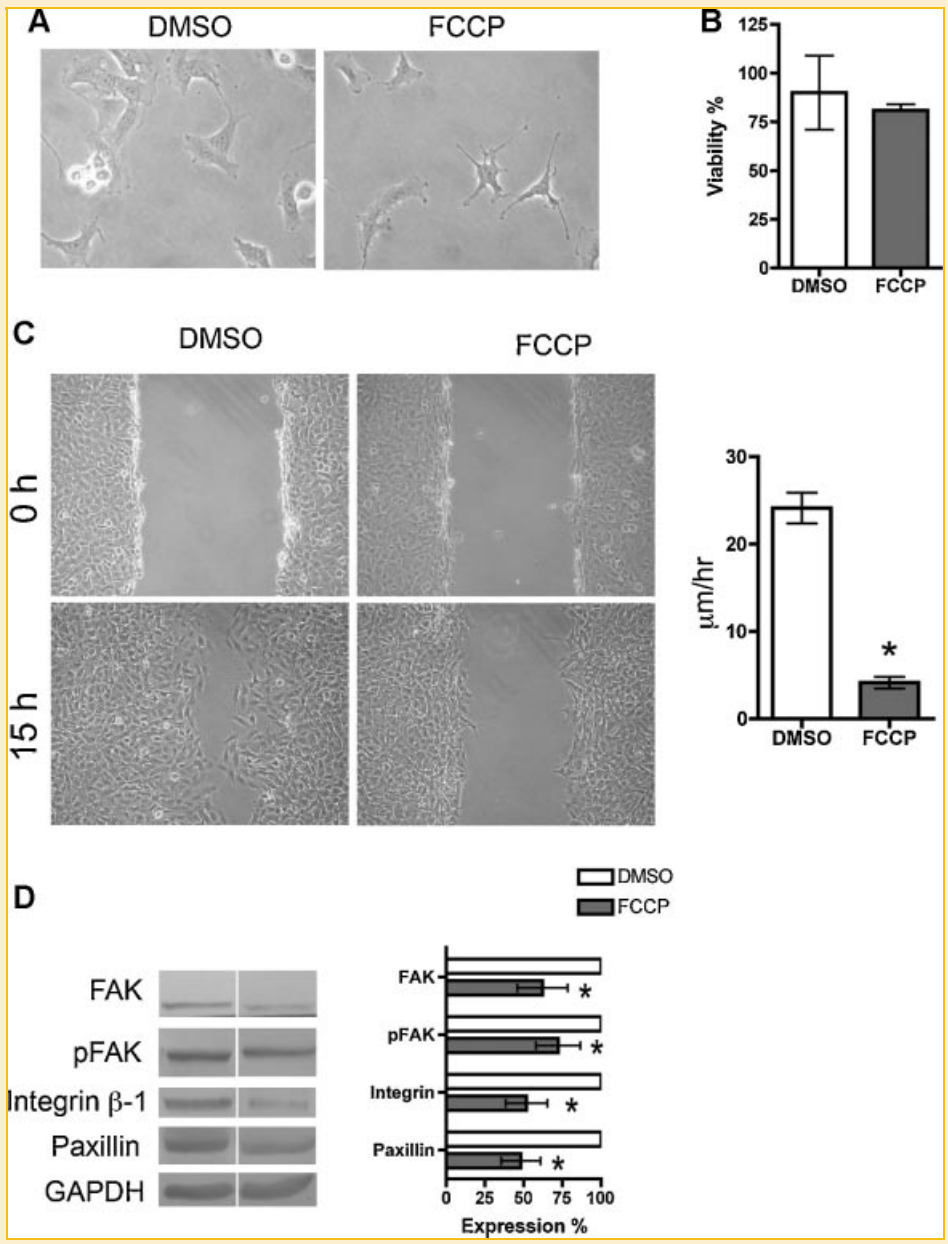

Fig. 6. Mitochondria uncoupling with FCCP recapitulates the effects of rottlerin. A: Phase photomicrographs of CGTH W-2 cells cultured for 24 h in growth medium with 10 μM FCCP or 0.1% DMSO. B: Viability. The viability of CGTH W-2 cells cultured for 24 h in with or without 10 μM FCCP was assessed with MTT assay (n = 3). C: Wound-healing assay. Cells treated with 10 μM FCCP reduced cleft-closing after 15 h incubation (n = 3); $^{*}P < 0.05$. D: Western blot analysis of focal adhesion proteins in CGTH W-2 cells treated with DMSO or 10 μM FCCP for 24 h. GAPDH was used as the loading control (n = 3); $^{*}P < 0.05$.

in the migration arrest induced by rottlerin. Finally, we showed the mechanism of rottlerin was not through inhibition of PKCδ, but rather uncoupling of mitochondria.

The notion that rottlerin is a selective PKCδ inhibitor is recently placed under scrutiny. Rottlerin does not block PKCδ in vitro, and was effective in the absence of PKCδ protein [Soltoff, 2007]. Instead, rottlerin is suggested to act as a mitochondria uncoupler [Soltoff, 2001]. Our finding that rottlerin failed to suppress the translocation of PMA-activated PKCδ and that mitochondria uncoupler FCCP recapitulated the rottlerin-induced migration inhibition support a mitochondria uncoupling role of rottlerin in CGTH W-2 cells.

The pharmacological effect of rottlerin in thyroid cancer is not fully studied. Rottlerin has been reported to antagonize the growth arrest induced by PMA in thyroid cancer cells [Koike et al., 2006], to inhibit ionizing radiation-induced JNK activation in thyrocytes [Mitsutake et al., 2001], and to suppress cisplatin-provoked ERK phosphorylation in transformed thyroid cells [Urso et al., 2005]. Our study is the first to define the migration-inhibitory effects of rottlerin in thyroid cancer cells.

We showed that rottlerin inhibits integrin β1 in CGTH W-2 cells. Integrins regulate cell motility in various cancer types [Brakebusch et al., 2002]. Integrin β1 upregulation is associated with increased follicular thyroid cancer invasion [Demeure et al., 1992]. Our group has shown that integrin β1 perturbation with specific antibodies leads to disturbed focal adhesion in CGTH W-2 cells [Chen et al., 2006]. Another group demonstrated rottlerin reduced renal cell carcinoma migration in an integrin β1-dependent manner [Brenner et al., 2008]. How rottlerin interacts with integrin β1, however, remains elusive.

Upon activation of integrin β1, FAK is rapidly recruited and its Tyr397 residue autophosphorylated [Mitra et al., 2005]. In follicular thyroid cancer, FAK is overexpressed [Kim et al., 2004], which is consistent with invasive behaviors [Owens et al., 1996]. Our data showing decreased FAK and pFAK after rottlerin intervention is in support of a critical role of FAK in regulating the migration of thyroid cancer.

Active pFAK leads to phosphorylation of paxillin. Paxillin is an important focal adhesion molecule that recruits structural and signaling molecules in order to regulate migration and cell spreading [Schaller, 2001; Carragher and Frame, 2004]. This signaling cascade results in turnover of focal adhesions and downstream increase in migration. In the present study, we found decreased protein levels of total and phosphorylated paxillin, and the staining pattern of phosphorylated paxillin was weak and diffuse at focal adhesions. The decrease in FAK and paxillin levels might explain the altered morphology and decreased motility after rottlerin treatment.

Focal adhesion activation is terminated by proteasomal degradation of FAK and paxillin [Carragher and Frame, 2004; Mitra et al., 2005]. Recent studies demonstrated a role of rottlerin in proteasome-mediated degradation pathways [Basu et al., 2008]. Our preliminary data showed MG132, a proteasome inhibitor, potently inhibited the downregulation of FAK caused by rottlerin (data not shown), suggesting the ubiquitin-mediated proteosomal degradation may underlie the rottlerin-induced focal adhesion deactivation.

RhoA and Rac-1 are Rho GTPases that organize the actin cytoskeleton. It is generally agreed that the Rho GTPases are controlled by the integrin/FAK/paxillin/Rho signal cascade [Mitra et al., 2005]. In this study, only GTP-bound form of RhoA (active form) was identified by RhoA effector protein (rhotekin)-beads, and the active Rac by PAK (p21 activated kinase 1)-beads. We noted that the activity of RhoA and Rac-1 did not decrease as much as their expression. This suggests that rottlerin exerts greater impact on the expression than on the activation of these two proteins. Transcriptional, translational, or post-translational modification (e.g., ubiquitin-proteasomal degradation) [Rolli-Derkinderen et al., 2005; Doye et al., 2006] might be involved in this decreased expression of RhoA and Rac-1. Both RhoA and Rac-1 have been reported to be degraded by the ubiquitin-proteasomal pathway [Lerm et al., 2002; Rolli-Derkinderen et al., 2005], and inactive forms of RhoA are more sensitive than active form to the degradation [Rolli-Derkinderen et al., 2005]. The decreased amounts of RhoA and Rac-1 might be due to degradation induced by rottlerin. Taken together, our results clearly demonstrate that rottlerin treatment inhibits the migration of CGTH W-2 cells by disrupting focal adhesions in a manner independent of PKCδ. Thus, rottlerin is a potential anti-cancer agent for thyroid tumors.

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