The anandamide analog, Met-F-AEA, controls human breast cancer cell migration via the RHOA/RHO kinase signaling pathway

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Abstract

The endocannabinoid system regulates cell proliferation and migration in human breast cancer cells. In this study, we showed that a metabolically stable analog of anandamide, 2-methyl-2′-F-anandamide (Met-F-AEA), inhibited the RHOA activity and caused a RHOA delocalization from the cell membrane to cytosol determining a decrease in actin stress fibers. Overexpression of a dominant negative of RHOA activity and treatment of these cells with a RHO-associated protein kinase (ROCK) inhibitor, Y 27632, mimicked Met-F-AEA effects on actin organization and cell migration. We suggest that the inhibitory effect of Met-F-AEA on tumor cell migration could be related to RHOA-ROCK-dependent signaling pathway.

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Introduction

Metastasis is a multifactorial process that includes the acquisition of a motile and invasive phenotype. During progression to a metastatic phenotype, tumor cells undergo a series of changes that begin with loss of contact inhibition and increased motility, allowing them to migrate from the primary tumor site, invade distant organs, and induce neo-vascularization resulting in metastasis (Chambers *et al*. 2002). Many of these changes are associated with dynamic actin reorganization and activation of signaling pathways through transmembrane receptors (Oft *et al*. 1998, Seasholtz *et al*. 1999, Muller *et al*. 2001, Roymans & Slegers 2001, Vivanco & Sawyers 2002). Members of the small guanosine triphosphatase (GTPase) family control cell adhesion and motility through reorganization of the actin cytoskeleton and regulation of actomyosin contractility (Yoshioka *et al*.1998, Etienne-Manneville & Hall 2002). Both RHOA (Fritz *et al*. 1999) and the related RHOC (Clark *et al*. 2000) are expressed at a relatively higher level in metastatic tumors, and their expression levels positively correlate with the stage of the tumors. However, mutations in the *RHO* gene have not yet been found in human tumors; rather, the overexpression of RHOA in the cell facilitates its translocation from the cytosol to the plasma membrane, where its activation results in stimulation of the actomyosin system, followed by cellular invasion both *in vitro* and *in vivo* (Yoshioka *et al*. 1999). One of the target molecules of RHO is the family of RHO-associated serine–threonine protein kinases (ROCK; Leung *et al*. 1995), which also participates in cell-to-substrate adhesions, stress fiber formation, and stimulation of actomyosin-based cellular contractility (Narumiya *et al*. 1997). In particular, several breast cancers contain large amounts of RHOA proteins, whereas RHOA was hardly or not detectable in adjacent normal tissue. In addition, the progression of breast tumors from WHO grade I to grade III is accompanied by a significant average increase in RHOA protein levels (Van Aelst & D'Souza-Schorey 1997, Hall 1998, Fritz *et al*. 1999, Yoshioka *et al*. 1999, Kamai *et al*.

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2001, 2003, Horiuchi *et al*. 2003). The endogenous cannabinoid system, consisting of the cannabinoid CNR1 and CNR2 transmembrane receptors G-protein coupled, their endogenous ligands (endocannabinoids), and the proteins that regulate endocannabinoid biosynthesis and degradation, is an almost ubiquitous signaling system involved in the control of cell fate. The endocannabinoids (anandamide, AEA, and 2-arachidonoyglycerol, 2AG) have been shown to inhibit the growth of tumor cells in culture and animal models by modulating key cell signaling pathways (Bifulco *et al*. 2006, Mackie & Stella 2006). We have also shown that a metabolically stable anandamide analog, 2-methyl-2′-F-anandamide (Met-F-AEA), arrests the growth of K-ras-dependent tumors, induced and/or already established *in vivo*, and it inhibits the formation of metastatic nodules in the Lewis lung carcinoma model, these effects being largely mediated by cannabinoid CNR1 receptors (De Petrocellis *et al*. 1998, 2000, Bifulco *et al*. 2001, Bifulco & Di Marzo 2002, Portella *et al*. 2003). Moreover, it has been observed that cannabinoid receptors activation is able to inhibit vascular endothelial cell migration and to downregulate the expression and the activity of matrix metalloproteinase-2, a proteolytic enzyme involved in tissue remodeling during angiogenesis and metastasis (Pisanti *et al*. 2007). Anandamide inhibits the migration of human colon carcinoma cells (SW480) through a CNR1-dependent mechanism (Joseph *et al*. 2004). Interestingly, it was recently found that increasing endogenous 2-AG, by blocking its metabolism, inhibits invasion of androgen-independent prostate cancer (PC-3 and DU-145) cells. The anti-invasive effect of endogenous 2-AG relies, once again, on CNR1 activation and consequent inhibition of the adenylyl cyclase and protein kinase A (PKA) pathway (Nithipatikom *et al*. 2004). Nevertheless, the molecular mechanism of the cannabinoids anti-metastatic effect has not been fully explored. Recently, we described that Met-F-AEA treatment is able to inhibit cell migration of the highly invasive and metastatic breast cancer cell lines, MDA-MB-231, as tested in migration assays with type IV collagen, the major component of the basement membrane. This effect is mediated by CNR1 receptor and it is accompanied by a remarkable decrease in phosphorylation of focal adhesion kinase (FAK) and CSK protein, two tyrosine kinases involved in the loss of adhesion and invasion (Grimaldi *et al*. 2006). In this study, we further investigated the molecular mechanism of anti-metastatic potential of Met-F-AEA by studying the effect of this agonist of CNR1 receptor on RHOA activity and actin organization.

Materials and methods

Drugs and cell culture

Met-F-AEA was purchased from Sigma. The selective CNR1 antagonist, SR141716, was kindly provided by Sanofi-Aventis (Montpellier, France). Toxin B was purchased from Calbiochem (La Jolla, CA, USA), the inhibitor of RHO kinase, Y 27632 from Sigma, GGTI-298 was purchased from Calbiochem. Monoclonal antibody to RHOA and polyclonal antibody to CNR1 receptor were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The human breast carcinoma cell line MDA-MB-231 was maintained in RPMI 1640 culture medium (Gibco BRL Life Technologies) supplemented with 10% inactivated fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were cultured in a humidified environment containing 5% CO_2 and held at a constant temperature of 37 °C.

Separation of particulate and cytosolic fractions

Cells were grown to subconfluency (60–70%), then lysed by ice-cold lysis buffer (50 mmol/l HEPES (pH 7.5), 50 mmol/l NaCl, 1 mmol/l $MgCl_2$, 2 mmol/l EDTA, 10 mmol/l NaF, 1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) and centrifuged at 100 000 ***g*** for 30 min in micro-ultracentrifuge, and the supernatant was collected as the cytosolic fraction. Pellets were resuspended, and membrane proteins homogenized in 150 µl lysis buffer containing 2% Triton X-114. The homogenate was centrifuged at 800 ***g*** for 10 min. The supernatant (particulate fraction) and pellet (detergent insoluble particulate fraction) were collected separately. Whole cell, cytosolic, and particulate fraction proteins were separated by SDS-PAGE.

Western blot analysis

Cells plated in 100 mm dishes in regular medium with serum were washed with ice-cold PBS and scraped into lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% Triton X100, 0.5% deossicolic acid, 10 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 10 mg/ml aprotinin). After removal of cell debris by centrifugation (14 000 ***g***, 5 min), about 50 µg of proteins were loaded on 12% SDS-polyacrylamide gels under reducing conditions. After SDS-PAGE, proteins were transferred to nitrocellulose membranes that were blocked with 5% milk (Bio-Rad Laboratories, Inc.) and incubated with anti-CNR1 antibody. After three washes, filters were incubated for 1 h at room temperature with horseradish peroxidase-conjugated

goat anti-rabbit secondary antibody. The membranes were then stained using a chemiluminescence system (ECL-Amersham Biosciences) and then exposed to X-ray film (Kodak).

Invasion assay

For chemotaxis assays, Boyden chambers (8 Am Transwell polycarbonate membrane, Costar, NY, USA) were coated with 50 μg/ml type IV collagen and blocked with 5 mg/ml BSA. Cells (1×10^5 cells) treated for 24 h with Met-F-AEA alone or in combination with toxin B (100 ng/ml) or Y 27632 (10 μM) were added to the upper compartment and incubated (at 37 °C for 4 h) in migration media RPMI in the presence or absence of FBS used as a chemotactic stimulus in the lower compartment. Chambers were washed with PBS, and migratory cells on the lower membrane surface were fixed with 3% formaldehyde (10 min). Cells were permeabilized with 0.2% Triton X-100 (5 min) and stained with Hoechst dye (5 min). The number of migrated cells was counted by a light microscope at 20× magnification: ten randomly chosen microscopic fields were counted per well, and the mean number was determined. Background levels of cells migrated in the absence of chemotactic stimuli (chemokinesis) were subtracted from all the experimental points.

RHOA activation assay

The amount of activated Rho(GTP-Rho) was determined using a Rho activation assay kit (Upstate Biotechnology, Inc., Lake Placid, NY, USA). The human MDA-MB-231 cells were grown to confluence and then placed in serum free for 24 h. After treatment, the cells were washed with ice-cold PBS. Cellular lysates were prepared according to the manufacturer's instructions.

Immunofluorescence

Cells plated on cover slips were washed once with PBS and fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, and incubated with PBS containing 4% BSA for 1 h at room temperature. F-actin was stained with tetramethyl rhodamine B isothiocyanate-labeled phalloidin (500 ng/ml; Sigma–Aldrich) to visualize filamentous actin. Cells were plated in 24-well plates on cover slips (Becton–Dickinson Labware, NY, USA). When they were 60 ± 80% confluent, they were treated with Met-F-AEA (10 μM for 15′, 3 and 24 h). After the incubation with various drugs, the cells were washed twice with PBS, fixed in 3.7% paraformaldehyde in PBS for 20 min and followed by two washes in 50 mM NH_4Cl for 10 min. Permeabilization was achieved by incubating the fixed cells in 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were then blocked in final dilution buffer (FDB) (1 mM $MgCl_2$, 1 mM $CaCl_2$, 5% fetal calf serum, and 2% BSA in PBS) for 30 min at room temperature. All primary and secondary antibody incubations were performed in FDB buffer for 1 h at room temperature. Cover slips were mounted on 50% glycerol in PBS and examined by using a Zeiss Laser Scanning Confocal Microscope (410 or 510).

Cell transfection

Plasmids were purified using the endotoxin-free plasmid midi prep kit (Qiagen).

For transient transfections, MDA-MB-231 cells were seeded in 6 cm plates and grown to 60–80% confluence in RPMI with 10% FBS without antibiotics and transfected the next day using FuGene reagent (Promega), according to the manufacturer's protocol, with a total of 2 μg/well of plasmid DNA. The cells were transfected with control vector (pcDNA3) or pcDNA3-N19RHOA provided by Dr Mario Chiariello (IEOS, CNR Napoli). Twenty-four hours after transfection, cells were prepared for immunofluorescence analysis and for wound-healing assay. For knocking down CNR1 protein expression, we used a 25-nucleotide small interfering RNA (siRNA) duplex (Dharmacon Research, Lafayette, CO, USA) designed for specific silencing of CNR1 (siRNA-CNR1) covered the sequence sense 5′-CCCAAGUGACGAAAA-CAUU-dTdT-3′ gently provided by Dr Patrizia Gazzerro (University of Salerno). We transfected cells using Lipofectamine (Invitrogen) and 30 nM siRNA for each transfection, in accordance with the manufacturer's protocol. An equal concentration of SilencerR Negative Control-1 siRNA (Ambion Inc. NY, USA) was used as negative control. The transfected MDA-MB-231 cells were serum starved and after 24 h assayed for RHOA activity. Specific silencing of targeted genes was confirmed by western blot analysis for CNR1 receptor.

Statistical analysis

All data were presented as means $\pm$ S.D. Statistical analysis was performed using one-way ANOVA analysis. In the case of a significant result in the ANOVA, Student's *t*-test was used for dose–response curves and Bonferroni's test for *post hoc* analysis for all other experiments. A P value less than 0.05 was considered statistically significant.

Results

Met-F-AEA inhibits RHOA activity and induced delocalization of RHOA from cell membrane

Since in a recent report we demonstrated that metabolically stable anandamide analog, Met-F-AEA at 10 μM inhibited the migration of MDA-MB-231 human breast cancer cells (Grimaldi *et al*. 2006), we hypothesized that this agonist of the CNR1 receptor could affect the activity of RND1/2/3 involved in the cell migration and actin organization of several cancer cells. To examine this hypothesis, we analyzed the level of the active GTP-bound form of RHOA by a pull-down assay with the RHO-binding fragment of rhotekin in cells treated with Met-F-AEA at 10 μM. The cells were developed in medium serum free for 24 h, and after treatment we collected the cells and performed the assay. As shown in Fig. 1A, a large proportion of the RHOA protein detected in MDA-MB-231 cells is present in an active form. Upon the stimulation of CNR1 receptor by Met-F-AEA, the level of GTP-RhoA was decreased after 15 min and strongly reduced after 1 h. The RHOA activity is restored in Met-F-AEA-treated cells in presence of SR141716, antagonist of CNR1 receptor. To evaluate the maximum effect of Met-F-AEA on RHOA activity, MDA-MB-231 cells were treated with increasing concentrations of Met-F-AEA from 2.5 to 10 μM, then they were collected and was performed a pull-down assay. As shown in Fig. 1D, the anandamide analog strongly reduced the RHOA activity in a dose-dependent manner. The reduction was statistically significant at 10 μM concentration compared with the control. Furthermore, in order to confirm that this effect is mediated by CNR1 receptor, we reduced the level of expression of CNR1 receptor by RNA interference. To specifically silence the *CNR1* gene, MDA-MB-231 cells were transfected with siRNA targeting *CNR1*

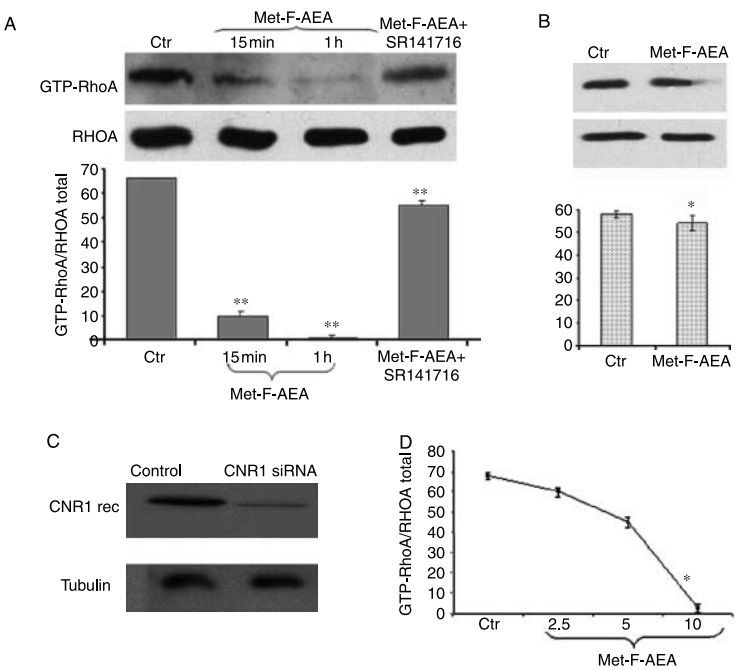

Figure 1 Inhibition of activity of RHOA by Met-F-AEA at 10 μM in MDA-MB-231 cells. (A) The upper western blots show the extent of the active form of RHOA (GTP-RhoA) and total RHOA in control cell and in cells treated with Met-F-AEA for 15 min, 1 h and in presence of Met-F-AEA plus SR141716 for 1 h. The lower panel summarizes the data of densitometric analysis of RHOA activity. RHOA activity is represented as the relative ratio of the density of GTP-RhoA against that of total RHOA. The assay is described in Materials and methods. (B) Effect of silencing mRNA (siRNA) for CNR1 receptor in MDA-MB-231 cells on RHOA activity. It is reported the RHOA activity assay in cell transfected with CNR1-siRNA untreated and treated with Met-F-AEA 10 μM for 1 h. The lower panel summarizes the data of densitometric analysis of RHOA activity. (C) Western blot analysis of CNR1 receptor of MDA-MB-231 cell lines expressing CNR1-siRNA or no silencing siRNA. Cells were analyzed with specific antibodies to CNR1 receptor. Decreased CNR1 receptor expression by CNR1-siRNA was present in MDA-MB-231 expressing CNR1-siRNA compared with the control cells no silencing siRNA. The target specificity of CNR1-siRNA was verified by using an anti-tubulin antibody. (D) Analysis of RHOA activity in cells treated with concentrations of Met-F-AEA from 2.5 to 10 μM for 1 h in comparison with untreated cells. The plot summarizes the data of densitometric analysis of RHOA activity. RHOA activity is represented as the relative ratio of the density of GTP-RhoA against that of total RHOA. The figures show a representative result of three independent experiments. All data were presented as the mean ± S.D. of three experiments (one-way ANOVA, $n=3$, $^{**}P<0.01$; $^{*}P<0.05$).

mRNA. Western blot analysis (Fig. 1C) showed that the protein level of CNR1 was markedly down-regulated by CNR1 siRNA at 24 h after administration of the siRNA, with reductions of 80% of protein expression of CNR1 receptor in MDA-MB-231 cell line compared with lysates transfected with no silencing siRNA. Incubation of CNR1-siRNA transfected cells with Met-F-AEA at 10 μM for 1 h did not affect the RHOA activity (Fig. 1B). More, because RHOA protein must be targeted to the plasma membrane for activation and full function, we examined the translocation of this protein from the cytosol to the cell membrane in MDA-MB-231 cells in the presence of Met-F-AEA at 10 μM, after separation of particulate and soluble fractions. In untreated cells without serum, most of RHOA protein was present on the cell membrane (particulate fractions; Fig. 2A, ctr and B), suggesting that in these breast cancer cells the overexpression of RHOA facilitated its translocation from the cytosol to the cell membrane as previously described (Fritz *et al*. 1999). Treatment of MDA-MB-231 cells with Met-F-AEA clearly decreased the amount of RHOA in the membrane fraction and accumulates in the cytosolic fraction in a time-dependent manner.

Anandamide induces reorganization of the actin cytoskeleton

As RHOA is involved in the regulation and assembly of contractile actin filaments (stress fibers), we studied the effect of Met-F-AEA on actin cytoskeleton in these cells by immunofluorescence confocal microscopy using phalloidin TRITC-conjugated. As shown in Fig. 3A, various stress fibers were detected in untreated cells. The treatment of these cells with Met-F-AEA at 10 μM caused a significant decrease in F-actin containing stress fibers, which was apparent within 15 min (Fig. 3C). After longer incubation times, many cells displayed a dense meshwork of unpolarized actin filaments around the cell periphery (3 and 24-h time point are shown in Fig. 3E and F). We also observed the intracellular distribution of RHOA with a specific monoclonal antibody and a secondary antibody FITC-conjugated. Interestingly, RHOA was present at the membrane periphery in untreated cells (Fig. 3B); while at 15 min of treatment with Met-F-AEA, the RHOA patches to the cell membrane are reduced (Fig. 3D). After 3 h, RHOA remained largely diffused in the cytoplasm mainly in the perinuclear region in confront to control sample and, at 24 h, the RHOA fluorescence-associated is equally widespread into cytosol. To confirm the role of RHOA in the actin organization, we transiently transfected the cells with a vector carrying a dominant negative of RHOA (N19RHOA) which antagonizes the RHO guanine nucleotide exchange factors (ARHGEFs),

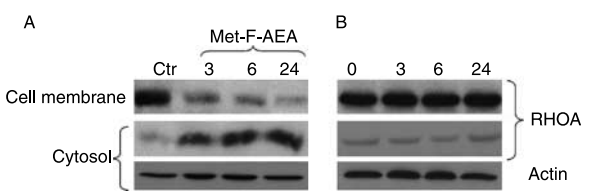

Figure 2 Met-F-AEA induced translocation of RND1 from the cell membrane to cytosol. Western blots indicate the particulate fractions (cell membrane) and soluble fractions (cytosol) of MDA-MB-231 cells treated with Met-F-AEA at 10 μM for 3, 6, and 24 h (A) and untreated cells in serum-free media (B), harvested at time points indicated. Equal amounts of protein were loaded on each lane. Immunoblot analyses were performed using specific anti-RHOA, anti-actin antibodies. The figure shows a representative blot of three independent experiments.

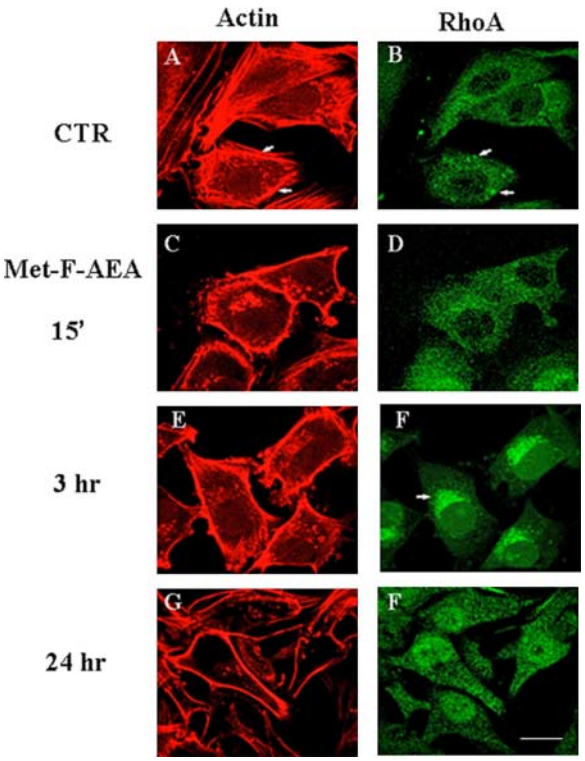

Figure 3 Stress fibers and subcellular distribution of RHOA. (A) The cells were grown on cover slips without (ctr) or with Met-F-AEA at 10 μM for 15 min, 3 and 24 h at 37 °C and fixed with paraformaldehyde. Stress fibers were revealed with phalloidin TRITC-conjugated under permeabilized conditions. RHOA was revealed with a monoclonal antibody anti-RHOA and a FITC-conjugated secondary antibody. The arrows in (A) display actin stress fiber and in (B) indicate the submembrane localization of RHOA. In (F) the RHOA localization is in proximity of cell nucleus (arrow). Bar, 10 μm. Full colour version of this figure available via http://dx.doi.org/10.1677/ERC-08-0030.

when compared with cells transfected with empty vector. As shown in Fig. 4, in cell transfected with empty vector (mock), the actin is organized in stress fibers, while in that with vector carrying N19RHOA we observed a significant reduction in actin stress fiber as how in Met-F-AEA-treated cells (Fig. 3E, C and G). In addition, because ROCK, the immediate downstream effector of RHOA, has been clearly implicated in cancer migration, we examined the effect of a selective inhibitor of ROCK I and II, Y 27632 (Ishizaki *et al*. 2000) on actin organization. Cells treated with this ROCK inhibitor at 10 μM reduced actin stress fibers (Fig. 4A). We previously reported that Met-F-AEA inhibited MDA-MB-231 cell migration on type IV collagen (Grimaldi *et al*. 2006), in order to verify the involvement of RND1 signaling pathway in the Met-F-AEA effect on cell migration, we used Toxin B of *Clostridium difficile*, which glycosylates threonine residue of RHOA causing the inactivation of small RND1 (Just *et al*. 1995), and Y 27632, a highly specific inhibitor of ROCK (also termed RHO-associated kinase, RHO-kinase), major effectors of RHOA (Ishizaki *et al*. 2000) alone and in combination with Met-F-AEA in chemotaxy assay. Data obtained in the absence of chemotactic-factor gradient (chemokinesis) were shown in Fig. 5. We observed that Y 23762 10 μM and Toxin B (100 ng/ml) inhibited cell invasion and the combination of Met-F-AEA and Y 23762 or toxin B did not show any additivity (Fig. 5).

Mevalonate (MVA) induces stress fibers reversion

In addition to GDP/GTP cycling, RND1/2/3 function is also believed to be critically dependent on post-translational modification by isoprenoid lipids. RND1/2/3 terminate in a COOH-terminal CAAX tetrapeptide sequence motif. This sequence signals for covalent attachment of an isoprenoid lipid group. These modifications (prenylation) promote the association of RND1/2/3 with plasma and intracellular membranes. The MVA pathway produces isoprenoids lipids such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate which in turn post-translationally modify the carboxy termini of many signaling molecules, such as RND1/2/3. Most RND1/2/3 are geranylgeranylated (Adamson *et al*. 1992, Collisson *et al*. 2002, Solski *et al*. 2002, Sebti 2005, McTaggart 2006). In order to ascertain the role of RHOA prenylation for its activity, we examined the ability of the MVA, central precursor of all isoprenoid lipids, to restore cytoskeletal changes in Met-F-AEA-treated cells. Figure 6E illustrates the recovery of actin stress fibers in response to MVA supplementation of Met-F-AEA-treated cells. The MVA supplementation recovered the subcellular distribution of RHOA in comparison with cells treated with the agonist alone, interestingly RHOA fluorescence-associated was mainly distributed in patches in proximity of the plasma membrane (Fig. 6F). Furthermore, we evaluated the hypothesis that the MVA supplementation to Met-F-AEA-treated cells could recover the cell migration by chemotaxis assay. As shown in Fig. 7A, the supplementation of MVA recovered the migration of Met-F-AEA-treated cells. In order to demonstrate

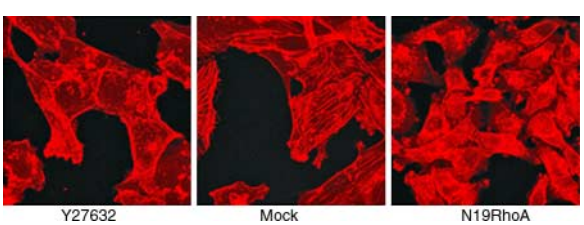

Figure 4 Actin organization in cell treated with Y 27632 for 24 h, in cells transfected with vector carrying dominant negative of RHOA (N19RHOA), and in cells transfected with empty vector (Mock). In control cells (mock), the actin is organized in stress fibers. Stress fibers formation was inhibited in cells treated with Y 27632 and cells transfected with vector carrying dominant negative of RHOA (N19RHOA). A representative of the three experiments is shown. All the immunofluorescence samples were observed using a Laser Scan Confocal microscope as described in Material and methods. Bar, 10 μm. Full colour version of this figure available via http://dx.doi.org/10.1677/ERC-08-0030.

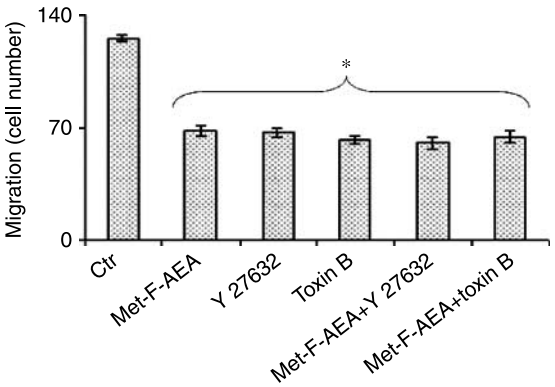

Figure 5 Effect of blocking of RHOA signaling pathway on cell migration. Cells were incubated with Met-F-AEA (10 μM) alone or in combination with Toxin B (100 ng/ml) or Y 27632 (10 μM) and plated in the upper compartment of Boyden chambers coated with type IV collagen. For chemotaxis assay, cells were pre-treated 24 h with the indicated substances and then plated in the upper compartment of Boyden chambers coated with type IV collagen. After 4 h at 37 °C, migratory cells in the lower chamber were stained and counted under a light microscope. Shown are the mean ± S.D. values of triplicates from at least four independent experiments. The background represented by the number of migrated cells in absence of the chemo-attractant factor (chemokinesis) was subtracted from each experimental point (ANOVA, $^*P<0.05$).

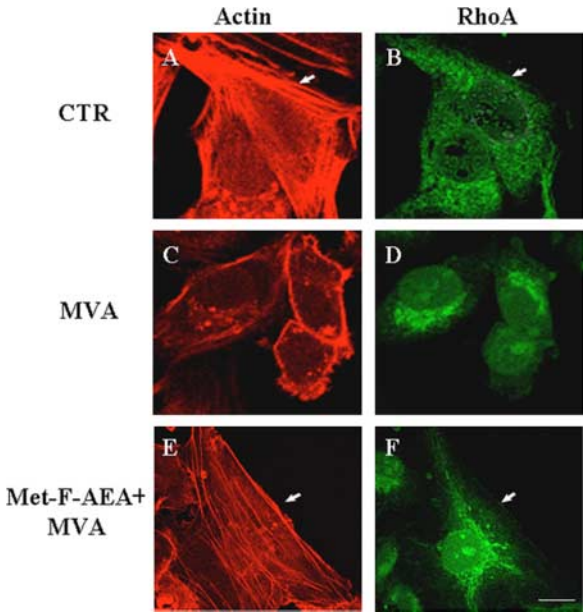

Figure 6 Effect of supplementation of mevalonic acid (MVA) at 700 μM in Met-F-AEA-treated cells. In untreated cells, the actin is organized in stress fibers (arrow in panel A). Arrow in panel B shows the submembrane localization of RHOA. In cells treated with Met-F-AEA at 10 μM, the actin is not organized in stress fibers (panel C) and RHOA is localized in perinuclear region (panel D). The MVA supplementation at the Met-AEA-treated cells recovered the subcellular distribution of RHOA and actin stress fibers. The arrows in panel E and F display the submembrane localization of RHOA and stress fibers in proximity of cell membrane. A representative of three experiments is shown. Full colour version of this figure available via http://dx.doi.org/10.1677/ERC-08-0030.

the role of geranylgeranylation of RHOA in cell migration of MDA-MB-231 cells, we used GGTI-298 (Kusama *et al*. 2003, 2006), an inhibitor of geranylgeranyltransferase I, enzyme that catalyzes the RHOA prenylation. GGTI-298 at 10 μM markedly attenuated the *in vitro* invasive capacity of MDA-MB-231 cells. Moreover, the MVA did not recover the effect of Met-F-AEA on cell migration in presence of GGTI-298 (Fig. 7A), suggesting that the geranylgeranylation of RHOA has an important role in cell migration of these breast cancer cells. In order to confirm the role of RHOA activity in cell migration of MDA-MB-231, we performed a RHOA activity assay for each treatment. As shown in Fig. 7B, the RHOA activity was inhibited in cells treated with Met-F-AEA and GGTI-298.

Discussion

Metastasis is the direct cause of mortality in most cancer patients. Metastatic process requires the abrogation of cell–cell contacts, the remodeling of cell matrix interactions, and finally the movement of the cell mediated by actin cytoskeleton. Many of these components are controlled by members of Ras superfamily of GTP-binding proteins that includes RND1/2/3 subfamily. All aspects of cellular motility and invasion, including cellular polarity, cytoskeleton organization, and transduction of signals from the outside environment are controlled through an interplay between the RND1/2/3. Once activated, RHOA triggers a complex set of signal transduction pathways that include both the RHO-associated coiled-coil containing protein kinase (ROCK) activation pathway, which is responsible for actin polymerization required for cell locomotion (Zhong *et al*. 2005). In the past, we proved that CNR1 receptor mediates the inhibition of migration of MDA-MB-231, a highly invasive human breast cancer cell line, in response to Met-F-AEA, through a reduction of tyrosine phosphorylation of FAK and CSK protein. Because this pathway interconnected with RHOA/RHO kinase pathway for the regulation of cell migration (Frame 2004), we supposed that this agonist by CNR1 receptor could affect the RND1/2/3 activity and the downstream signaling. In the present study, we show that Met-F-AEA could alter endogenous RHO signaling in MDA-MB-231 cells and this alteration might decrease metastatic potential *in vitro* of these cells. We observed that CNR1 stimulation by Met-F-AEA affects the distribution and activation state of RHOA and consequently actin organization. We described that Met-F-AEA at 10 μM caused an inhibition in the GTPase activity of RHOA that it is recovered in the presence of antagonist of CNR1 receptor suggesting that this effect is CNR1-receptor

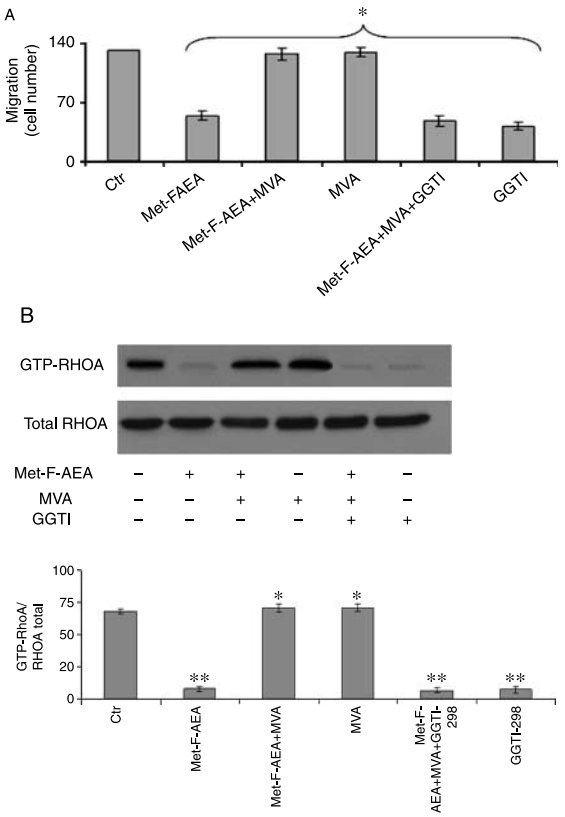

Figure 7 (A) MDA-MB-231 cells treated with Met-F-AEA at 10 μM alone and in combination with mavalonic acid (MVA) at 700 μM and GGTI-298 at 10 μM, and untreated cells were plated in the upper compartment of Boyden chambers coated with type IV collagen. After 4 h at 37 °C, migratory cells in the lower chamber were stained and counted under a light microscope. Shown are the mean ± S.D. values from three independent experiments (ANOVA, $*P<0.05$). (B) The western blot of levels of the active form of RHOA (GTP-RhoA) and total RHOA in control cell and in cells treated for 1 h with the compounds used for the invasion assay. The lower panel summarizes the data of densitometric analysis of RHOA activity. RHOA activity is represented as the relative ratio of the density of GTP-RhoA against that of total RHOA. The figure shows a representative blot of three independent experiments. All data were presented as the mean ± S.D. of three experiments (one-way ANOVA, $n=3$, $**P<0.01$, $*P<0.05$).

mediated. Moreover, in order to confirm the receptor-dependent mechanism, we inhibited specifically the CNR1 receptor signaling using chemically synthesized siRNAs to achieve direct gene silencing for CNR1 receptor. After verifying the specificity and efficacy of CNR1-siRNA for knocking down CNR1 protein expression, we tested the RHOA activity upon the CNR1 stimulation by Met-F-AEA. We reported that the silencing of CNR1 receptor reversed the Met-F-AEA effect on RHOA activity. Consequently, treatment of MDA-MB-231 cells with Met-F-AEA attenuated the translocation from the cytosol (inactive form) to the plasma membrane (active form) in a time-dependent manner (Fig. 2). This event could cause a significant reduction of stress fibers formation determining the loss of traction forces dependent on actin cytoskeleton required for cell motility. Indeed, we showed a reduction of the stress fibers after treatment of MDA-MB-231 cells with Met-F-AEA at 10 μM The RHOA distribution also changed upon the CNR1 stimulation by Met-F-AEA; in untreated cells, fluorescence RHOA-associated was in proximity of the plasma membrane, while in treated cells after 3 h RHOA was in perinulclear region and afterwards widespread into cytosol, suggesting that Met-F-AEA induced a delocalization of RHOA from cell membrane to the cytoplasm and this effect led to the disruption of skeleton actin stress fibers, as already described (Vincent *et al.* 2001). To gain further insight into the role of RHOA in the migration of breast cancer cell, we examined the effect of the overexpression of dominant negative RHOA (N19RHOA) on actin organization and cell migration. The overexpression of dominant negative of RHOA markedly inhibited both stress fibers organization and the migration cell (Figs 4C and 5B). Moreover, the treatment of these cells with Toxin B from *C. difficile*, that inactivates the RHO family members by glycosylation of threonine residue of these proteins, caused the similar effects on cell migration (Fig. 5A). In addition, we demonstrated that the inhibition of ROCK by Y 23762 inhibited the transmigration of MDA-MB-231 cancer cells through Matrigel-coated membranes (Fig. 6) and it reduced stress fibers formation (Fig. 4C). Taken together, data showed in this study suggest that RHOA/ROCK signaling could be involved in the maintenance of actin organization and induction of migration in the MDA-MB-231 cells and that this CNR1 agonist could affect this pathway. Although various RND family members (such as RHOA, RHOB, and RHOC) are highly homologous and could be affected by Met-FAEA, our present data suggest that the inhibition of RHOA/ROCK signaling is critical for suppressing metastatic activity in the treated cells. These data support results by Pillè who used anti RHOA siRNA to inhibit the RHOA synthesis in MDA-MB-231 cells. *In vitro*, these siRNAs inhibited cell proliferation and invasion more effectively than conventional blockers of RHO cell signaling (Pillé *et al.* 2005). In conclusion, RND1/2/3 are well-characterized proteins whose activity depends on post-translational modification by isoprenoids compounds. The pharmacologic inhibition of RHO geranylgeranylation produced similar inhibition of RHO localization and signaling that include the reorganization of the actin cytoskeleton, and focal adhesion assembly suggesting that the prenylation of RHOA is critical event for cancer cell invasion

(Denoyelle *et al*. 2001, Schmidmaier *et al*. 2004, Zhong *et al*. 2005). Here, we observed that the supplementation of MVA, central precursor of isoprenoid compounds, to cell treated with Met-F-AEA restored F-actin stress fibers, intracellular distribution of RHOA, and cell migration. These results suggest that the stimulation of CNR1 receptor by Met-F-AEA could affect the pathway of isoprenoid synthesis. To confirm this hypothesis, we showed that *in vitro* invasion of breast cancer cells was inhibited potently by GGTI-298, inhibitor of geranyl transferase I GGTase-I, enzyme of pathway of isoprenoids that it catalyzes the geranylgeranylation of RHOA. Moreover, we observed that the RHOA activity was inhibited in cells treated with GGTI-298 while was recovered in cells treated with Met-F-AEA in presence of MVA. These data strongly suggest that the inhibition of the geranylgeranylation of RHOA is critical for the RHOA activity and consequently for cancer cell invasion. Finally, the crucial point of the anti-metastatic action of Met-F-AEA could be related to the inhibition of the RHOA prenylation, causing the disruption of RHOA localization to the membrane, required to modulate its interaction with upstream and downstream signaling components, thereby attenuating RHO's ability to promote invasion. This report represents the first study to disclose a unique underlying mechanism of Met-F-AEA against tumor metastasis.

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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