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Inhibition of adipogenesis by RGD-dependent disintegrin

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Abstract

Adipogenesis plays a central role in obesity development. The processes of adipogenesis include migration, adhesion, proliferation and survival of preadipocytes and differentiation to mature adipocytes. Many of these biological functions are related to integrins. Here, we found that snake venom-derived arginine–glycine–aspartic acid (RGD)-containing disintegrin inhibited adipogenesis. Rhodostomin but not rhodostomin RGD mutants (RGE-Rn and AKGDWN-Rn) caused the detachment of primary cultured preadipocyte. Furthermore, rhodostomin also inhibited focal adhesion of preadipocyte, including the inhibition of the expression of focal adhesion kinase (FAK) and FAK phosphorylation, assembly of vinculin and reorganization of actin cytoskeleton. Cell viability of preadipocytes was decreased after rhodostomin treatment in a concentration-dependent manner. The results of flow cytometric analysis showed that rhodostomin induced cell apoptosis. In addition, chromatin condensation was observed in DAPI staining. The increase of Bax expression and activation of capsase-3 was detected following rhodostomin treatment. Addition of dexamethasone, IBMX and insulin induced differentiation of preadipocytes into mature adipocytes and treatment of cells with rhodostomin during the initial 3 days showed less mature adipocytes following 9–10 days of differentiating period. The triglyceride content and gene expression of peroxisome proliferators-activated receptor gamma (PPARγ) and leptin also decreased in response to the treatment of rhodostomin. These results suggest that disintegrin inhibits processes of adipogenesis and may be developed to treat obesity.

Keywords: Adipocyte; Disintegrin; Integrin; Adipogenesis

1. Introduction

Growth of adipose tissue involves both hyperplasia and hypertrophy of the adipocytes [1]. Adipocytes play a major role in energy homeostasis. They store energy in the form of lipid during plethora of food and release the stored energy in response to nutritional needs or inadequacies [1]. Adipocytes are not only involved in energy regulation, but also perform an endocrine function by secreting hormones and factors, which are involved in the regulation of food intake, immune response, reproduction, insulin sensitivity, and vascular and skeletal growth [2,3]. Although adipocytes perform important physiological functions, excessive body deposition of fat can result in obesity. Obesity increases the risk for many diseases especially heart disease, hypertension, stroke, cancer and diabetes [4]. Because the growth of adipose tissue can be due to both hyperplasia and hypertrophy of adipocytes, the process of adipocyte differentiation and its regulation has been the focus of several studies [5,6].

During adipocyte differentiation, cells convert from a fibroblastic morphology to a spherical shape, and dramatic changes occur in cytoskeletal components and the level and type of extracellular matrix (ECM) components. Many of the studies on the effect of cytoskeletal and ECM components in adipocyte differentiation predate the characterization of adipocyte transcription factors [5]. Integrins are receptor αβ heterodimers with overlapping specificity toward ECM components [7]. Integrin-mediated cell–ECM interactions promote the assembly of cytoskeletal and signaling molecule complexes at sites called focal adhesions. Integrin-focal adhesion kinase signaling complexes have been implicated in the regulation of

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anchorage-dependent cell survival. Integrins expressed in most tissues, including insulin-sensitive adipose and muscle cells, bind to particular extracellular matrix proteins. The key biological functions of integrins, including cell migration and adhesion, are mediated in part by focal adhesion kinase (FAK), $pp125^{FAK}$ [8]. There is evidence that signaling pathways initiated by integrins synergize functionally with those triggered by growth factors [9,10]. Recent data imply that insulin potently augments α5β1 integrin-mediated cell adhesion of insulin receptor-expressing CHO cells, while signaling via this integrin in turn enhances insulin receptor kinase activity and tyrosine phosphorylation and formation of complexes containing insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3K) [11]. These studies suggest that integrin signaling pathway may play an important role in adipogenesis.

Disintegrins are a family of low-molecular-weight, RGD containing peptides that bind specifically to integrins αIIbβ3, α5β1 and αvβ3 expressed on platelets and other cells including vascular endothelial cells and some tumor cells [12]. In addition to their potent antiplatelet activity, studies of disintegrins have revealed new uses in the diagnosis of cardiovascular diseases and the design of therapeutic agents in arterial thrombosis, osteoporosis and angiogenesis-related tumor growth and metastasis [13]. Therefore, disintegrin is a useful tool to investigate the role of integrin in the adipocyte and adipogenesis. In the present study, we investigated the action of rhodostomin, a disintegrin purified from venom of *Calloselasma rhodostoma*, and found that disintegrin induced preadipocyte apoptosis and inhibited adipogenesis. Our results suggest that disintegrin may be developed to treat obesity.

2. Materials and methods

2.1. Materials

Rhodostomin was purified from venom of *C. rhodostoma* [14].

Collagenase, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, Oil Red O, propidium iodide, 4′,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), triglyceride reagent, FITC-conjugated phalloidin and mouse anti-human vinculin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbeco's Modified Eagle's Medium (DMEM) and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). All culture plates were purchased from TPP (Switzerland).

2.2. Cell isolation and primary culture of rat preadipocyte

Fibroblastic preadipocytes were isolated from adipose tissue using a method modified from Zhao et al. [15]. The subcutaneous inguinal fat deposits from male Sprague–Dawley rats weighed 200–250 g was removed under sterile conditions and the lymph nodes were carefully removed. The stroma-vascular cells were obtained from the minced fat tissues by collagenase (1 mg/ml) digestion at 37 °C for 45 min in Hank's balanced salt solution under agitation. The resulting cell suspension was filtered through a sterile 100-μm nylon mesh and centrifuged at 500 g for 10 min. The floating top layer of mature adipocytes was removed, the pellets were washed several times and the suspended cells were filtered through a 25-μm nylon filter to eliminate residual aggregates. The cells obtained were cultured in DMEM medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and amphotericin (100 U/ml, 100 μg/ml and 2.5 μg/ml, respectively; Gibco). When the cells reached confluence, the differentiation was initiated by the addition of dexamethasone (1 μM), IBMX (0.5 mM) and insulin (10 μg/ml) in DMEM supplemented with 10% fetal bovine serum. After 72 h, the differentiation medium was replaced by DMEM supplemented with 10% fetal bovine serum plus insulin only. This medium was changed every 2 days. Ten days later, cells were regarded as differentiated by morphologic criteria when, after acquiring a round shape, their cytoplasm was filled with multiple lipid droplets (assessed by Oil Red O staining).

2.3. Assay of cell detachment

Cell detachment was determined by the assay of viability of adherent cells using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay as described previously [16]. After incubation with rhodostomin for 48 h, cultures were washed with PBS to remove non-adherent cells. MTT (0.5 mg/ml) were then added to each well and the mixture was incubated for 2 h at 37 °C. Culture medium was then replaced with equal volume of DMSO to dissolve formazan crystals. After shaking at room temperature for 10 min, absorbance of each well was determined at 550 nm using a microplate reader (Bio-Tek, Winooski, VT).

2.4. Oil Red O staining

Cytoplasmic lipid droplets were stained with Oil Red O. Primary preadipocytes were rinsed three times with PBS and then fixed in 4% paraformaldehyde for 15 min. The cells were washed PBS and then stained with Oil Red O solution (0.35 g of Oil Red O in 100 ml of isopropyl alcohol) for 15 min at room temperature. Images were taken using an IX70 microscope (Olympus, Tokyo, Japan).

2.5. Measurement of triglyceride content

At the end of adipocyte differentiation, cells were lysed in lysis buffer (0.25 M sucrose, 1 mM sodium–EDTA,

5 mM Tris and 1 mM dithiothreitol, pH 7.4) and sonicated. Total triglyceride was assayed using Sigma Diagnostics Triglyceride Reagent. Results were expressed as total triglyceride content per experiment well.

2.6. Immunocytochemistry

Primary preadipocytes were grown on glass coverslips. Cultures were rinsed once with PBS and fixed for 30 s at room temperature in acetone. Cells were rinsed three times with PBS and then blocked with 4% BSA for 15 min. To examine the extracellular distribution of vinculin, cells were incubated with primary mouse monoclonal anti-vinculin (Sigma) in a dilution of 1:400 for 1 h at room temperature. After being washed three times with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG (1:150) for 1 h. For F-actin visualization, cells were incubated with FITC-conjugated phalloidin (1:200; Sigma) for 1 h at room temperature. Finally, cells stained with vinculin or actin were washed, mounted, and examined with a Leica TCS SP2 Spectral Confocal System.

2.7. Flow cytometric analysis of apoptosis

Primary preadipocytes in the presence or absence of rhodostomin were collected by centrifugation and fixed by 70% alcohol at 4 °C overnight. Alcohol was then removed by centrifugation and DNA of the cells was stained with propidium iodide staining solution (100 μg/ml propidium iodide, 0.1% Triton-X, 1 mM EDTA in PBS) in the presence of an equal volume of DNase-free RNase (200 μg/ml) for 45 min and analyzed by FACS Calibur (Becton Dickinson, USA).

2.8. Western blotting analysis

Primary preadipocyte were plated on six-well (35 mm) dishes. Cells incubated with rhodostomin for 2 or 24 h were collected, which includes detached and adherent cells, and lysed for 30 min at 4 °C with radioimmunoprecipitation assay (RIPA) buffer. Thirty micrograms of protein was applied per lane, and electrophoresis was performed under denaturing conditions on a 7% (for the detection of p-FAK and FAK) or 15% (for the detection of Bax and caspase-3) polyacrylamide-SDS gel and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were blocked with 4% BSA for 1 h at room temperature and then probed with anti-phospho-FAK (1:1000; Upstate Biotechnology, Lake Placid, NY), anti-FAK, anti-Bax or anti-caspase 3 (1:1000; Santa Cruz, CA, USA) for 1 h at room temperature. After three washes, the blots were subsequently incubated with goat anti-rabbit or goat anti-mouse peroxidase-conjugated secondary antibody (1:1000) for 1 h. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). For normalization purposes, the same blot was also probed with anti-ERK2 antibody (1:1000; Santa Cruz).

2.9. RT-PCR

Total RNA was isolated from cell using Trizol™ Reagent (MDBio, Taipei, Taiwan). Reverse transcription reaction was performed using 5 μg of total RNA and reversely transcribed into cDNA using oligo dT primer, then amplified using oligonucleotide primers as follows:

PPARγ [454 base pairs (bp)] TGGGTGAAACTCTGGGAGAT and CCATAGTGGAAGCCTGATGC [17];
Leptin (244 bp) CCTGTGGCTTTGGTCCTATCTG and AAGGCAAGCTGGTGAGGATCTG [18];
GAPDH (452 bp) ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA [19].

PCR was carried out at 94 °C for 30 s, at 65 °C for 30 s and 1 min at 68 °C for 34 cycles. The PCR products were run on 2% agarose gels and visualized by ethidium bromide. Quantitative data were obtained using a computing densitometer and ImageQuant Software (Molecular Dynamics).

2.10. Construction of rhodostomin D51E and P48A/R49K/M52W/P53N mutants

The expression of rhodostomin D51E (RGE-Rn) and P48A/R49K/M52W/P53N (AKGDWN-Rn) mutants in *Pichia pastoris* was accomplished by following protocols previously described [20]. The wild-type construct was used to produce D51E and P48A/R49K/M52W/P53N mutations using overlap extension PCR. The expression kit and the yeast transfer vector, pPICZαA, were purchased from Invitrogen. The structural gene of *Rho* was amplified by polymerase chain reaction (PCR) with sense primer 5′-GAATTCGAATTCCATCATCATCA TCATCATGGTAAGGAATG TGACTGTTCTT-3′ that has *Eco*R1 recognition and six histidine residues for facilitating purification. The antisense primer was 5′-CCGCGGCCGCGGTTAGTGG TATCTTGGACAGTCAGC-3′ with *Sac*II recognition and TTA stop codon. The PCR product was purified and then cloned into the *Eco*R1 and *Sac*II sites of yeast recombination vector, pPICZαA. The recombinant plasmid was transformed into *DH5α* strain, and the colony was selected by agar plate with low salt LB (1% of tryptone + 0.5% of yeast extract + 0.5% of NaCl + 1.5% of agar at pH 7.0) and 25 μg/ml of the antibiotic Zeocin. After the clone was confirmed by sequencing the insert, 10 μg of plasmid was digested with *Sac*I to linearize the plasmid. The linearized construct was transformed into the *Pichia* strain, X33, using a *Pichia* EasyComp kit from Invitrogen. The transformant integrated at the 5′ AOX1 locus by single crossover, and the colony was selected by

agar plate with YPDS (1% yeast extract, 2% peptone, 2% glucose, 2% agar and 1 M Sorbitol) and 100 μg/ml Zeocin. PCR analysis was used to analyze *Pichia* integrants to determine if the gene had integrated into the *Pichia* genome. We picked the highest protein expression clone from a number of clones with multicopies of gene insertion.

2.11. Statistics

The values given are means ± S.E.M. The significance of difference between the experimental group and control was assessed by Student's *t*-test. The difference is significant if the *p*-value is <0.05.

3. Results

3.1. Rhodostomin caused the detachment of primary cultured preadipocyte

To understand the effect of rhodostomin on preadipocytes, we cultured the primary preadipocytes isolated from rat adipocyte tissue. When cells formed a confluent monolayer, the culture medium was replaced with serum-free medium in the presence of absence of rhodostomin (10 μg/ml). Following incubation for 48 h, rhodostomin caused the detachment of primary preadipocytes (Fig. 1A). The detachment of cells was quantitatively evaluated by MTT assay. After washout of the detached cells, the adherent cells were assayed by MTT reaction. As shown in Fig. 1B, the adherent primary preadipocytes were markedly

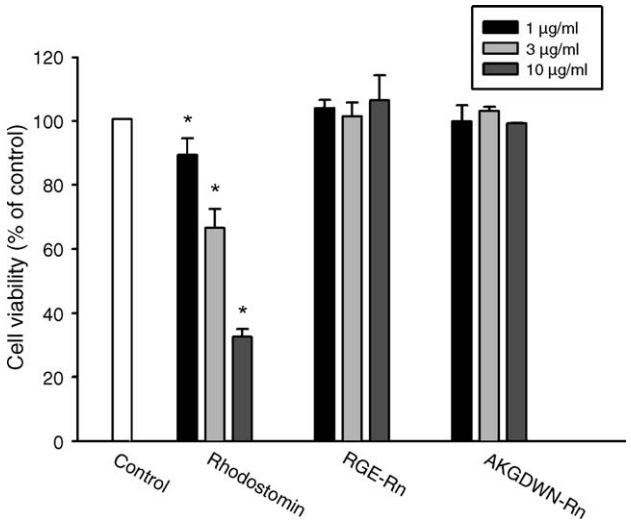

Fig. 2. Effect of rhodostomin mutants on cell detachment in primary preadipocytes. Primary preadipocytes were treated with various concentrations of different rhodostomin mutants and wild-type rhodostomin for 48 h, and cell viability was measured by MTT assay. Results are expressed as percentage of control (mean ± S.E.M.) ($n = 4$). $^{*}p < 0.05$ as compared with control.

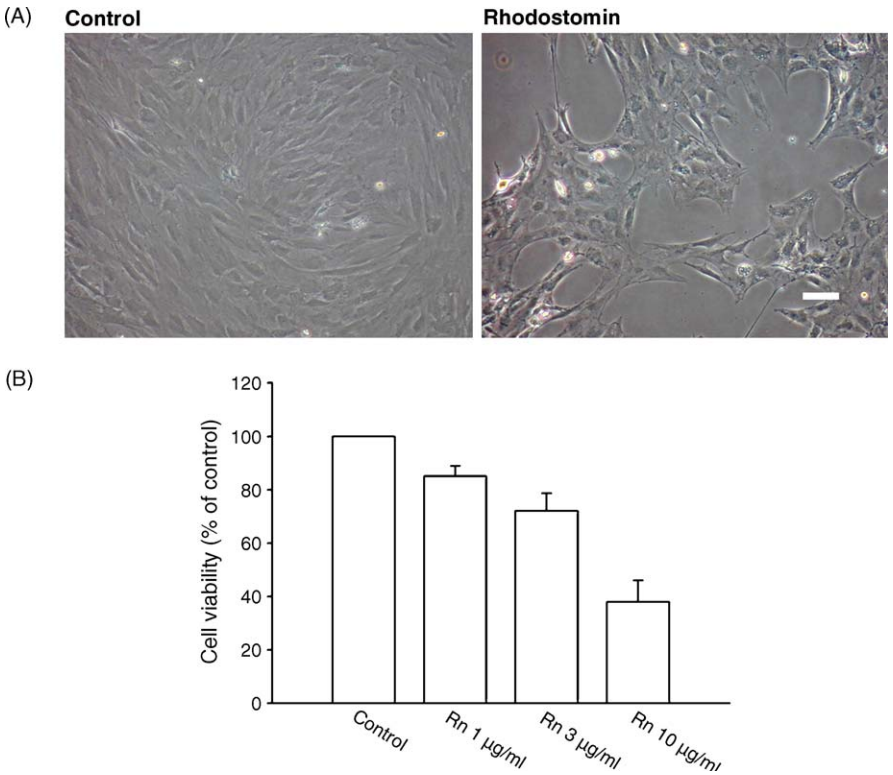

Fig. 1. Detachment of primary preadipocytes induced by rhodostomin. (A) Primary preadipocytes treated with rhodostomin (Rn) (10 μg/ml) for 48 h were photographed under a microscope. Bar, 50 μm. (B) MTT assay was used to assay the viability of adherent cells and make a quantitative analysis of the detachment of cells indirectly. Results are expressed as percentage of control (mean ± S.E.M.) ($n = 4$). Note that rhodostomin caused cell detachment in a dose-dependent manner.

reduced by rhodostomin in a concentration-dependent manner. Previous studies have indicated that the RGD motif is very important for rhodostomin to bind αIIbβ3, α5β1 and αvβ3 integrin on the cell surface [12]. To further investigate the relationship between RGD containing rhodostomin and preadipocytes, we compared wild-type rhodostomin and rhodostomin mutants on cell detachment. In these rhodostomin mutants, some of the amino acid in RGD motif had been mutated and the affinity to αvβ3 integrin also decreased [20]. For example, the affinity of rhodostomin RGE-Rn and AKGDWN-Rn mutants to αvβ3 is decreased by >685 and 3287-fold and α5β1 affinity is decreased by >25 and >503-fold, respectively. Primary preadipocytes were then incubated with mutants of RGE-Rn and AKGDWN-Rn for 48 h and found that they did not cause cell detachment (Fig. 2). These results suggest that rhodostomin causes the detachment of preadipocytes by blocking αvβ3 integrin in a RGD-dependent manner.

3.2. Rhodostomin altered expression of FAK, vinculin and organization of actin cytoskeleton

Activated integrin will recruit signal transduction molecules and cytoskeleton to form focal adhesion, which plays an important role in cell adhesion. Since rhodostomin induced cell detachment, we further investigated whether cytoskeleton and focal adhesion complex components of FAK and vinculin were affected by rhodostomin. As shown in Fig. 3A, incubation of primary preadipocytes with low concentrations of rhodostomin for 2 h significantly decreased FAK phosphorylation but not total expression of FAK. However, treatment of primary preadipocytes with rhodostomin at a higher concentration of 10 μg/ml for 24 h, the FAK expression was significantly decreased (Fig. 3B). Vinculin is another critical component in focal complex. It was found that immunofluorescent vinculin assembled on the edge of cells in normal condition (Fig. 4A), which disappeared in rhodostomin-treated cells (Fig. 4B). In addition, actin cytoskeleton also associated with focal complex to control cell adhesion and maintain cell shape [21]. With FITC-phalloidin staining, control cells showed typical long straight stress fibers running across the cell body (Fig. 4C). However, stress fibers were decreased in primary preadipocytes incubated with rhodostomin for 24 h (Fig. 4D). These results indicated that rhodostomin influenced the assembly of focal adhesion complex and actin cytoskeleton. Rhodostomin-induced detachment of preadipocytes may result from the changes of these focal adhesion components.

3.3. Rhodostomin induces apoptosis of primary preadipocyte

Since rhodostomin induces cell detachment, we then examined whether these cells undergo apoptosis. The primary preadipocytes were treated with rhodostomin (10 μg/ml) for 48 h. Floating and adherent cells were harvested and

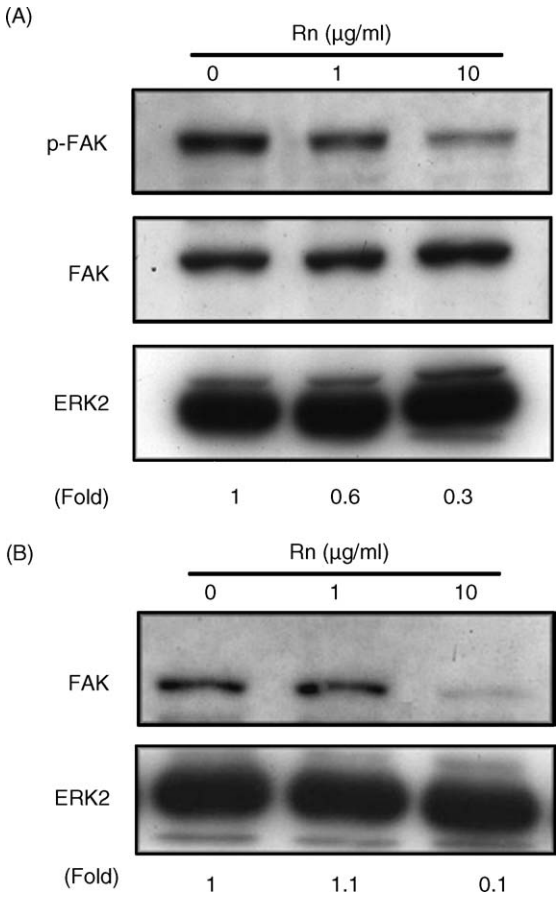

Fig. 3. Inhibition of rhodostomin on FAK phosphorylation and FAK expression in primary preadipocytes. (A) Primary preadipocytes treated with different concentrations of rhodostomin for 2 h were collected and analyzed by Western blot. Note that p-FAK decreased in response to rhodostomin treatment. (B) High concentration of rhodostomin decreased FAK expression in primary preadipocytes after 24 h treatment. The ERK2 protein level was considered as an internal control. The results were representative of three independent experiments.

analyzed by flow cytometry, respectively. The floating cells had DNA degradation, as judged by the appearance of a sub-G1 peak during cell cycle progression (Fig. 5A and B). This was confirmed by immunocytochemistry staining of DNA content. Treatment of primary preadipocytes with rhodostomin (10 μg/ml) for 24 h resulted in a significant nuclei with separate globular structures (apoptotic bodies, arrows), whereas nuclei of untreated cells had no such apoptotic bodies (Fig. 5C). Treatment with rhodostomin for 48 h caused apoptosis of primary preadipocytes in a concentration-dependent manner (Fig. 6A). We then investigated whether the expression of Bax was affected during rhodostomin-induced apoptosis. As shown in Fig. 6B, incubation with various concentrations of rhodostomin increased Bax expression in preadipocytes. To examine the involvement of caspases in rhodostomin-induced apoptosis, we analyzed the cleavage of procaspase. Preadipocytes treated with various concentration of rhodostomin caused caspase-3 activation (i.e., the appearance of cleaved caspase-3 with a molecular mass ~20 kDa) in a concentration-dependent manner (Fig. 6C). These results suggest that Bax expression

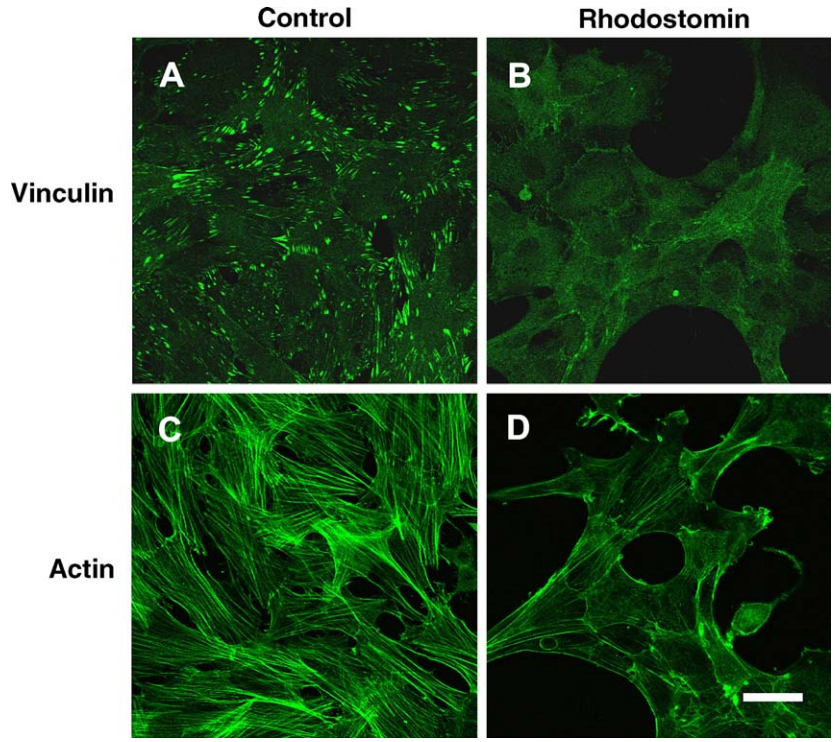

Fig. 4. Effect of disintegrin on vinculin distribution and actin cytoskeleton. Primary preadipocytes plated on coverslips and incubated in the absence (A) or presence (B) of rhodostomin (Rn) (10 μg/ml) for 24 h were fixed and stained with vinculin. Note that control cells have more cluster formation of vinculin on the edge of cells than rhodostomin-treated cells. Actin filaments were stained with FITC-phalloidin. Note that stress fibers were observed in control cells (C) and markedly decreased in rhodostomin-treated cells (D). Bar, 40 μm.

and activation of caspase-3 may be involved in rhodostomin-induced cell detachment and apoptosis.

3.4. Rhodostomin decreases adipogenesis in primary cultured preadipocytes

Differentiation of primary preadipocytes was induced by the addition of dexamethasone, IBMX and insulin. As shown in Fig. 7A, accumulation of lipid droplets, a hallmark of differentiated adipocytes, was visualized with Oil Red staining. Treatment with rhodostomin for the initial 3 days inhibited adipogenesis in a concentration-dependent manner when the adipogenesis was evaluated by triglyceride content 12 days after differentiating period (Fig. 7). Adipocyte differentiation is accompanied by complex changes in the pattern of gene expression [5]. To determine

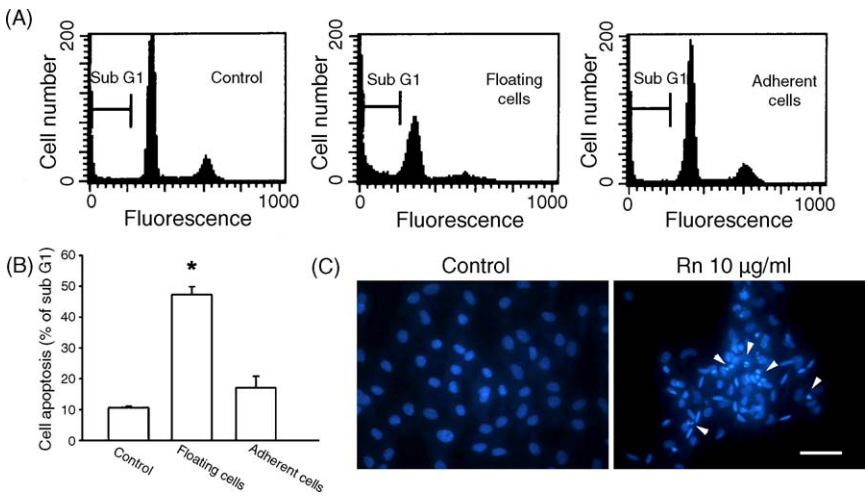

Fig. 5. Apoptosis of preadipocytes induced by rhodostomin. (A) Primary preadipocytes were incubated with rhodostomin (10 μg/ml) for 48 h. Floating and adherent cells were harvested and analyzed by flow cytometry. The sub-G1 region represents cells undergoing apoptosis-associated DNA degradation and is expressed as a percentage with respect to the entire cell cycle. The quantitative data are shown in (B). (C) Primary preadipocytes were exposed to rhodostomin (Rn) (10 μg/ml) for 24 h and the adherent cells were then stained with DAPI. Disintegrin-treated cells show more small nuclei with chromatin condensation as indicated by arrowheads. Bar, 50 μm. $^{*}p < 0.05$ as compared with control.

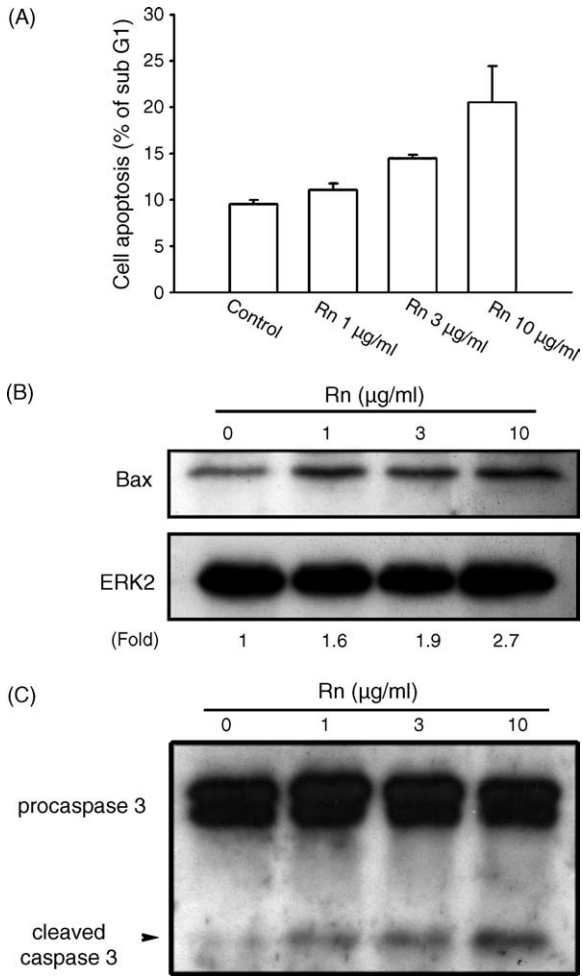

Fig. 6. Bax expression and procaspase-3 activation induced by rhodostomin treatment. (A) Primary preadipocytes treated with various concentrations of rhodostomin (Rn) for 48 h were analyzed by flow cytometry. A fractional DNA content (sub-G1) was recorded and expressed as mean ± S.E.M. Note that rhodostomin caused cell apoptosis in a dose-dependent manner. Cells incubated with various concentrations of rhodostomin for 24 h were then collected and analyzed by Western blotting. Each blot is representative of three independent experiments. Note that Bax (B) and cleaved caspase-3 (C) were increased at high concentration of rhodostomin.

if the attenuation of cytoplasmic lipid accumulation by rhodostomin involves alteration in expression of genes encoding key adipogenic transcription factors, the mRNA expression of PPARγ and leptin was explored. Primary cultured preadipocytes were treated with various concentration of rhodostomin during the initial 3 days, and total RNA was extracted 12 days after differentiating period. It was found that rhodostomin decreased the mRNA level of both PPARγ and leptin in a concentration-dependent manner (Fig. 8).

4. Discussion

Obesity is characterized by increased adipose tissue mass that results from an increase of both number (hyperplasia) and size of fat cells (hypertrophy) [22]. The number of adipocytes present in an organism is determined to a large degree by the adipocyte differentiation process, which generates mature adipocytes from fibroblast-like preadipocytes. Many of the molecular details of this process are unknown. Recently, a number of promising extra- and intracellular inhibitors of fat cell formation have been identified. Understanding the balance between positive and negative regulators of adipogenesis has important health-related implications for anti-obesity medical therapy [23]. Here, we found that RGD-dependent disintegrin of rhodostomin inhibits process of adipogenesis, including survival of preadipocyte and differentiation to mature adipocytes, and may be developed to treat obesity.

RGD-dependent disintegrins specifically bind to integrins of αIIbβ3, α5β1 and αvβ3 expressed on platelets and other cells including vascular endothelial cells and some tumor cells [12]. It has been shown that disintegrin induced apoptosis of endothelial cell and inhibited angiogenesis both in vitro and in vivo [24]. Furthermore, disintegrin causes proteolysis of β-catenin, apoptosis of endothelial cells and regulating cell viability [25]. However, the action of disintegrin on adipogenesis is little known. In this study, we demonstrated that rhodostomin caused cell detachment and induced apoptosis of primary preadipocytes. Several lines of evidence indicate that cell detachment is a prerequisite for rhodostomin-induced apoptosis of preadipocytes. First, rhodostomin induced cell detachment, as shown by observation under a microscope and quantitative analysis of the detachment of cells by MTT reaction. Second, the flow cytometry analysis shows that rhodostomin induced cell apoptosis in floating cells but not in adherent cells. Previous study has shown that rhodostomin induced apoptosis of endothelial cell via cell detachment, which was induced probably through the blockade of integrin αvβ3 [25]. The RGD motif of rhodostomin is the major binding site for αvβ3 integrin on the cell surface [20]. Rhodostomin mutants, for which RGD motif is replaced by RGE or KGD peptides, did not cause the cell detachment. These results suggest that rhodostomin induced cell detachment by the blockade of αvβ3 integrin. Rhodostomin caused the detachment of primary cultured preadipocyte in different culture plates, i.e., TPP (Switzerland), Costar (USA), Falcon (France) and glass coverslip from Assistant (Germany). Furthermore, rhodostomin also induced the detachment of primary cultured preadipocyte in the presence of serum. These results suggest that the detachment of primary preadipocyte induced by rhodostomin is not particular to the specific culture plates or culture medium.

Integrins act as a link between extracellular matrix, cytoskeletal proteins and actin filaments [7]. Integrin clustering and cell adhesion induce the phosphorylation of FAK [26]. Phosphorylation of regulatory proteins in adhesion plaques, such as talin, paxillin, vicullin, integrins and FAK seems to control the architecture of ECM. Previous studies reported that human cultured endothelial cells

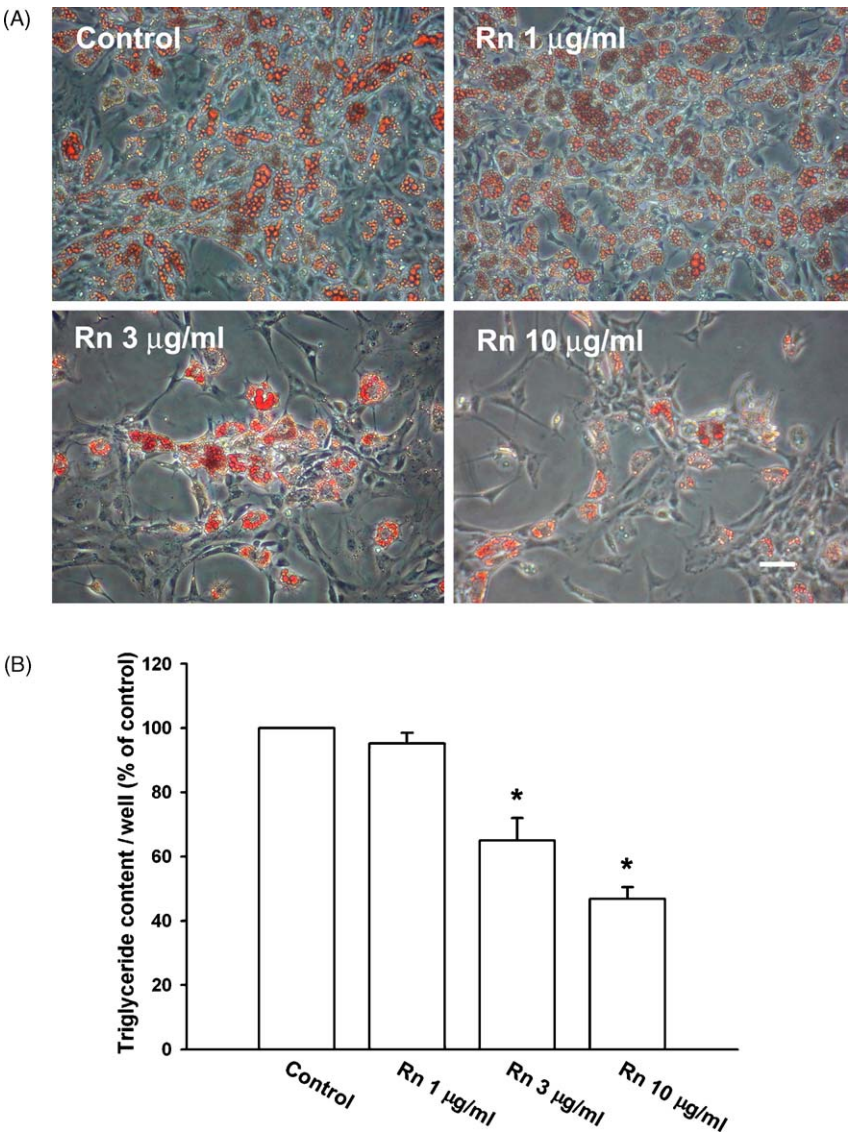

Fig. 7. Inhibition of rhodostomin on the differentiation of mature adipocytes. (A) Differentiation of primary preadipocytes was induced by the addition of dexamethasone, IBMX and insulin. Cells were co-incubated with different concentrations of rhodostomin (Rn) during the initial 3 days. The cells were fixed and stained with Oil Red to visualize the lipid content 12 days after differentiation, or lysed for the assay of triglyceride (B). Results are expressed as a percentage of control (mean ± S.E.M.) ($n = 5$). $^{*}p < 0.05$ as compared with control. Bar, 50 μm.

undergo apoptosis when the cells were detached from matrix by an addition of disintegrin [24]. Here, we show that rhodostomin also inhibits tyrosine phosphorylation of $pp125^{FAK}$ and FAK expression in preadipocytes. In addition, rhodostomin also influenced the assembly of focal adhesion complex and caused rearrangement of actin cytoskeleton. Therefore, preadipocytes are sensitive to disintegrin to be detached via the blockade of adhesion molecules, especially integrins, leading to anoikis and apoptosis.

Integrins are essentially important for cell adhesion to ECM, ligation of integrins induces a cascade of intracellular signals, regulates gene expression, and contributes to the mechanisms of proliferation, differentiation and cell survival [27]. Ligation of integrin αvβ3 on human umbilical vascular endothelial cell (HUVEC) suppressed p53 activity, blocked p21WAF1/CIP1 expression and increased the Bcl-2/Bax ratio, thereby promoting cell survival [28]. On the other hand, prevention of cell attachment to matrix, thereby blocking the integrin-induced signals and inducing apoptotic cell death [29]. It is found here that rhodostomin increases Bax expression and procaspase-3 activity of primary preadipocytes, which may also be involved in the process of apoptosis.

It has been reported that plasminogen activator inhibitor-1 (PAI-1) regulates adipogenesis through the interaction of vitronectin with its receptor, the αvβ3 integrin [30]. Treatment of preadipocyte suspension with rhodostomin markedly inhibited cell adhesion to vitronectin (data not shown). Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor regulating an array of diverse functions in a variety of cell types including the

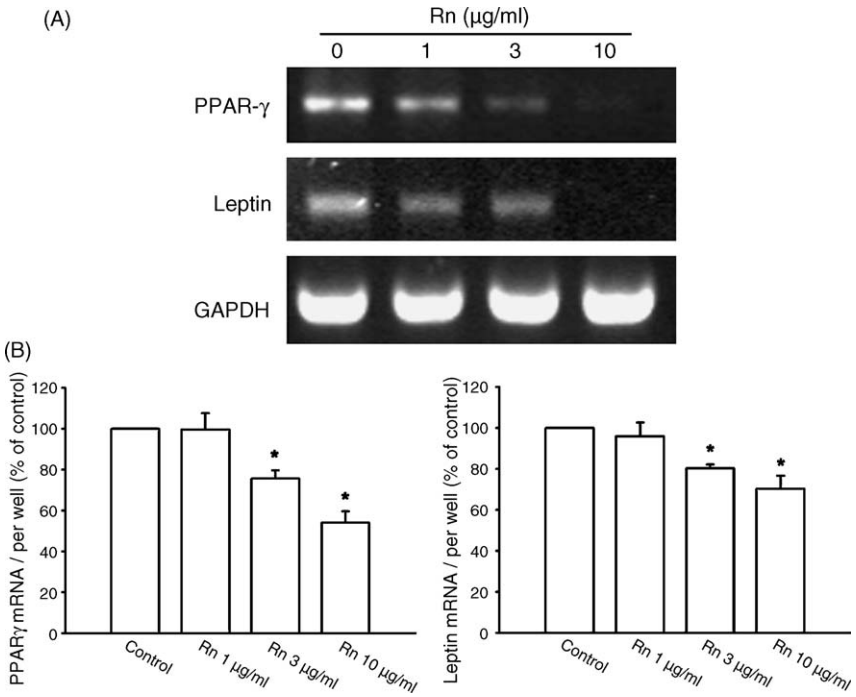

Fig. 8. Inhibition of rhodostomin on the mRNA expression of PPARγ and leptin in differentiating adipocytes. (A) Differentiation of primary preadipocytes was induced by the addition of dexamethasone, IBMX and insulin and treated with different concentration of rhodostomin (Rn) during the initial 3 days. Total RNA was extracted and mRNA levels of PPARγ and leptin were measured by RT-PCR 12 days after differentiation. The quantitative data were shown in (B). Results are expressed as a percentage of control (mean ± S.E.M.) ($n = 3$). $^{*}p < 0.05$ as compared with control.

development of adipose tissue, which involves coordinating expression of many hundreds of genes responsible for establishment of the mature adipocyte phenotype [31]. On the other hand, leptin is an important part of the lipostatic system because it signals the size of the energy reserves existing in the body and controls fuel mobilization and utilization [32]. It is found here that treatment with rhodostomin for the initial 3 days markedly inhibited adipogenesis evaluated both by triglyceride content and mRNA expression of PPARγ and leptin 12 days after differentiating period. Furthermore, rhodostomin also preferentially caused the detachment of adipocyte during initial 3 days of differentiating period (data not shown). Therefore, rhodostomin inhibited differentiation of preadipocyte into mature adipocyte through the reduction of preadipocyte number and cell viability during initial differentiating period. Recently, we found that ovariectomy for 6 weeks increased body weight (from 282.0 ± 6.0 to 368.0 ± 2.3 g, $n = 6$) in comparison with sham-operated rats (from 279.3 ± 4.8 to 338.4 ± 4.3 g, $n = 6$). However, intravenous administration of rhodostomin (0.3 mg/kg/day)) partially antagonized the increase of body weight caused by ovariectomy (from 282.6 ± 3.4 to 345.0 ± 7.3 g, $n = 7$). Whether the antagonism of rhodostomin is related to integrin blockade in adipocyte needs further investigation.

In conclusion, our results provide the first evidence to show that rhodostomin induced detachment and apoptosis of preadipocyte, which was caused by the blockade of integrin αvβ3. Furthermore, the changes of $pp125^{FAK}$ phosphorylation, arrangement of vicullin and actin cytoskeleton, Bax expression and the activation of caspase-3 are involved in the process of apoptosis. Rhodostomin thus inhibited adipogenesis from preadipocytes into mature adipocytes. These results suggest that RGD-dependent disintegrins inhibit process of adipogenesis and may be developed to treat obesity.

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