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H-89 potentiates adipogenesis in 3T3-L1 cells by activating insulin signaling independently of protein kinase A

Yoshiro Kato *, Nobuaki Ozaki, Tsutomu Yamada, Yoshitaka Miura, Yutaka Oiso

Department of Endocrinology and Diabetes, Field of Internal Medicine, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho Showa-ku Nagoya 466-8550, Japan

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Abstract

Among four kinds of protein kinase A (PKA) inhibitors tested, H-89 exhibited a unique action to remarkably enhance adipocyte differentiation of 3T3-L1 cells, whereas the other three PKA inhibitors, PKA inhibitor Fragment 14–22 (PKI), Rp-cAMP, and KT 5720, did not enhance adipocyte differentiation. H-85, which is an inactive form of H-89, exhibited a similar enhancing effect on adipocyte differentiation. H-89 also potentiated the phosphorylation of Akt and extracellular signal-regulated kinase (ERK) 1/2 in 3T3-L1 cells, which function as downstream signaling of insulin. Phosphoinositide 3-kinase (PI3K) inhibitor wortmannin and mitogen-activated protein kinase kinase (MEK) inhibitor PD 98059 suppressed both the H-89-induced promotion of adipocyte differentiation and the H-89-induced potentiation of phosphorylation of Akt and ERK1/2. Rho kinase inhibitor Y-27632 also promoted the phosphorylation of both Akt and ERK1/2 and enhanced adipocyte differentiation, although its effect was somewhat less than that of H-89. Even when cells were treated with a mixture of Y-27632 and H-89, the additive enhancing effects on both the insulin signaling and adipocyte differentiation were not detected. Therefore, it is suggested that the major possible mechanism whereby H-89 potentiates adipocyte differentiation of 3T3-L1 cells is activation of insulin signaling that is elicited mostly by inhibiting Rho/Rho kinase pathway.

Keywords: 3T3-L1 cells; Adipogenesis; H-89; Protein kinase A; Rho kinase; Phosphoinositide 3-kinase; Akt; Ras; Extracellular signal-regulated kinase 1/2

Introduction

Expanded adipose tissue mass or obesity is associated with insulin resistance and increases the risk for many clinical conditions including diabetes, hypertension, coronary heart diseases and some forms of cancer (Hausman et al., 2001). Adipose tissue is now considered to function not only as an energy storage tissue but also as a major endocrine organ, producing a variety of bioactive substances so-called adipocytokines, such as leptin, tumor necrosis factor-α, plasminogen activator inhibitor-1, and adiponectin. These adipocytokines are involved in the pathophysiology of various obesity-linked disorders and biological processes that include immune and inflammatory reactions (Ouchi et al., 2003).

The murine 3T3-L1 cell line provides a useful *in vitro* model of adipocyte differentiation (Gregoire et al., 1998). 3T3-L1 cells are well known for their ability to undergo complete differentiation into mature adipocytes in response to hormonal stimulation typically by using insulin, dexamethasone (Dex), and 3-isobutyl-1-methylxanthine (IBMX) (Cowherd et al., 1999; MacDougald and Lane, 1995). The differentiated cells exhibit many of the morphological and molecular characteristics of adipocytes found in the white adipose tissue; these characteristics include accumulation of triglyceride, insulin-regulated metabolism, and expression of characteristic adipocyte genes, such as peroxisome proliferator-activator receptor γ (PPARγ) and adipocyte-specific lipid binding protein (aP2) (Bernlohr et al., 1985; Green and Kehinde, 1975).

It has been reported that insulin is a key factor in the process of differentiation of 3T3-L1 cells (Smith et al., 1988; Uehara et al., 1991, 1994). The insulin receptor, which is a tyrosine kinase, phosphorylates the insulin receptor substrate (IRS) proteins and Shc, resulting in the activation of Ras/extracellular signal-regulated

* Corresponding author. Tel.: +81 52 744 2142; fax: +81 52 744 2212.
E-mail address: y-kato@kde.biglobe.ne.jp (Y. Kato).

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kinase (ERK) 1/2 pathway (Taha and Klip, 1999). The phosphorylated IRS proteins activate phosphoinositide 3-kinase (PI3K) and its downstream targets, protein kinase B (PKB, also known as Akt) and p70 ribosomal protein S6 kinase (p70S6K) (Taha and Klip, 1999). In 3T3-L1 cells, both of these pathways are responsible for adipocyte differentiation (Bost et al., 2005; Kohn et al., 1996; Magun et al., 1996; Prusty et al., 2002; Tomiyama et al., 1995; Xu and Liao, 2004; Zhang et al., 1996). In addition, cAMP and glucocorticoids are generally considered necessary for the induction of adipocyte differentiation. However, the mechanism of adipogenesis remains to be clarified.

In our studies to assess the effect of PKA inhibitors on adipocyte differentiation of 3T3-L1 cells, we found that H-89 exhibited a unique action to remarkably enhance adipocyte differentiation, whereas the other PKA inhibitors tested did not exhibited such an action. H-89 is synthesized isoquinolinesulfonamide and has been reported to be a highly specific PKA inhibitor with little effect on the activity of protein kinase C and other protein kinases (Chijiwa et al., 1990). Recently, however, H-89 has been found to inhibit several protein kinases, including p70S6K and Rho-dependent protein kinase II (Davies et al., 2000; Leemhuis et al., 2002).

The purpose of the present study is to assess the enhancing effect of H-89 on adipocyte diffrentiation of 3T3-L1 cells and to elucidate the mechanism underlying its enhancing effect.

Materials and methods

Materials

Insulin, Dex, IBMX, wortmannin and protein kinase A inhibitor Fragment 14–22 (PKI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Y-27632, PD 98059 and KT5720 were from Calbiochem (San Diego, CA, USA). H-89 and H-85 were from Seikagaku Co. (Tokyo, Japan). Rp-cAMP was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polyclonal antibodies against ERK1/2, phospho-ERK1/2, Akt and phospho-Akt (Ser473) were from Cell Signalling Technology Inc. (Beverly, MA, USA).

Cell culture

3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml of penicillin in humidified atmosphere of 5% CO_2/95% air at 37 °C to confluence, and 2 days later the induction of adipocyte differentiation was started by replacement of the differentiation medium containing 1 μM Dex, 0.5 mM IBMX and 5 μg/ml insulin and incubated for 2 days, followed by additional 2 days of treatment with the medium containing 5 μg/ml insulin alone. Medium was re-

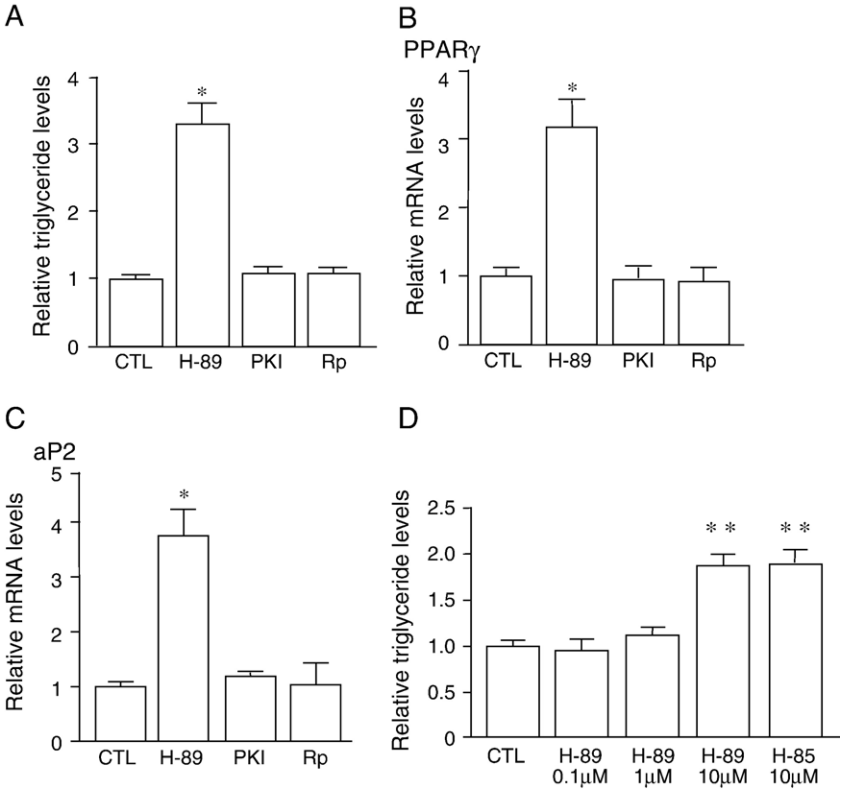

Fig. 1. Effects of PKA inhibitors on adipocyte differentiation of 3T3-L1 cells. (A) Triglyceride content in 3T3-L1 cells treated with various PKA inhibitors at day 12. Cells were treated with the differentiation medium in the absence (CTL) or presence of 10 μM H-89 (H-89), 10 μM PKI (PKI) or 100 μM Rp-cAMP (Rp) for 4 days after induction. The level of triglyceride content was expressed relative to that in the cells treated in the absence of PKA inhibitors (CTL). Effects of PKA inhibitors on the expression of PPARγ mRNA (B) and aP2 mRNA (C). Total RNA was extracted from 3T3-L1 cells treated with various PKA inhibitors and applied to quantitative real-time PCR with primers specific for PPARγ and aP2. 36B4 mRNA was used as internal control. The mRNA level was expressed relative to that in the control cells (CTL). (D) Triglyceride content in 3T3-L1 cells treated with various concentrations of H-89 and 10 μM H-85 at day 12. Cells were treated in the absence (CTL) or presence of 0.1 to 10 μM H-89 or 10 μM H-85 for 4 days after induction. The level of triglyceride content was expressed relative to that in the cells treated in the absence of H-89 or H-85 (CTL). Data were obtained from 3 independent experiments ($n=9$). Values are means±S.E. * indicates $p<0.001$ and ** indicates $p<0.05$.

placed every 2 days for the following 8 days. In the experiments to assess the effects of the materials to be tested, they were added into the differentiation medium and kept in the medium throughout the initial 4 days of adipocyte differentiation. The materials to be tested were used at the following concentrations; PKI (10 μM), Rp-cAMP (100 μM), H-89 (0.1 to 10 μM), KT 5720 (1 μM), H-85 (10 μM), PD 98059 (20 μM), wortmannin (1 μM), Y-27632 (10 μM).

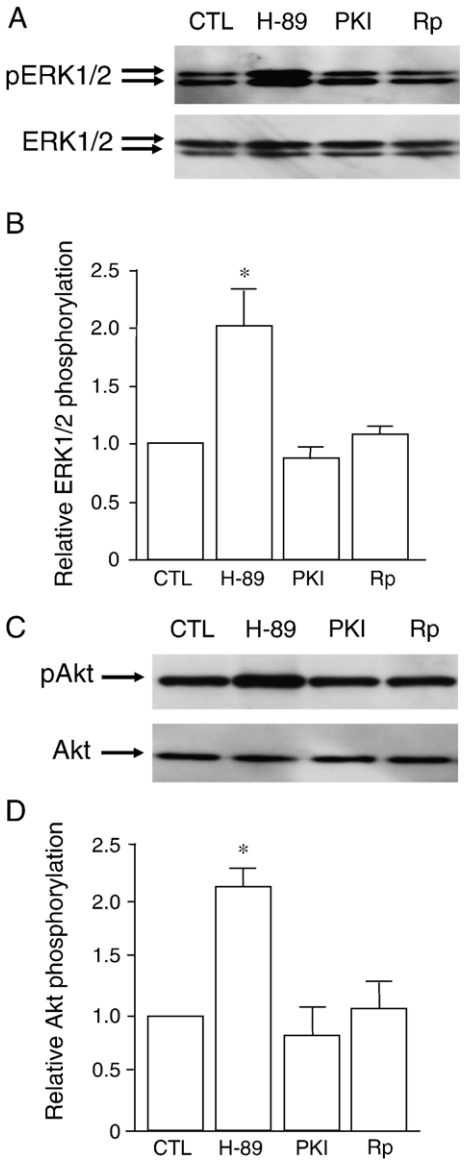

Fig. 2. Effects of PKA inhibitors on insulin signaling in 3T3-L1 cells. (A) Representative immunoblots of phospho-ERK1/2 and total ERK1/2. Cells were treated with the differentiation medium in the absence (CTL) or presence of 10 μM H-89 (H-89), 10 μM PKI (PKI) or 100 μM Rp-cAMP (Rp) for 10 min after induction. Cell lysates were analyzed for phospho-ERK1/2 (pERK1/2) and total ERK1/2 (ERK1/2) by immunoblotting using specific antibodies. (B) Relative ratio of phospho-ERK1/2 to total ERK1/2. The level of ERK1/2 phosphorylation was expressed relative to that in the cells treated in the absence of PKA inhibitors (CTL). (C) Representative immunoblots of phospho-Akt and total Akt. Cells were treated with the differentiation medium in the absence (CTL) or presence of 10 μM H-89 (H-89), 10 μM PKI (PKI) or 100 μM Rp-cAMP (Rp) for 10 min after induction. Cell lysates were analyzed for phospho-Akt (pAkt) and total Akt (Akt) by immunoblotting using specific antibodies. (D) Relative ratio of phospho-Akt to total Akt. The level of Akt phosphorylation was expressed relative to that in the cells treated in the absence of PKA inhibitors (CTL). Data were obtained from 3 independent experiments. Values are means± S.E. * indicates $p<0.05$.

Triglyceride assay

For measurement of triglyceride content, the cells were washed twice, harvested by scraping and homogenized with phosphate-buffered saline (PBS). Triglyceride content was measured using a Triglyceride E Test WAKO kit (Wako Pure Chemicals, Osaka, Japan).

Quantitative real-time reverse transcriptase-polymerase chain reaction (Quantitative real-time RT-PCR)

Total RNA was extracted from 3T3-L1 cells using the Trizol reagent (Life Technologies, Grand Island, NY, USA). Three μg of total RNA was reverse-transcribed using Transecriptor Reverse Transcriptase (Roche Molecular Biochemicals, Mannheim, Germany). After cDNA synthesis, quantitative real-time PCR was performed in 25 μl reactions containing SYBR Green I PCR Matrix (Eurogentec, Seraing, Belgium) using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reaction mixtures were incubated for initial denaturation at 95 °C for 10 min, followed by 40 cycles, each cycle consisting of 95 °C for 15 s and 60 °C for 1 min. The level of PPARγ and aP2 mRNAs was normalized to that of 36B4. Sequences of primers used in this study were described previously (Li et al., 2003).

Western blot analysis

3T3-L1 cells were grown in 35 mm dishes and serum-starved for 12 h before induction. The cells were washed 3 times with PBS and harvested in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5 mM Na_3VO_4) with protease inhibitors (Roche Molecular Biochemicals) 10 min after induction. Thirty micrograms of each soluble extract was subjected to SDS-PAGE, followed by transferring onto nitrocellulose membranes, followed by immunoblot analysis with respective antibodies against ERK1/2 (at a dilution of 1:1000), phospho-ERK1/2 (at a dilution of 1:1000), Akt (at a dilution of 1:1000) and phospho-Akt (at a dilution of 1:1000). The target protein was detected by ECL plus detection kit (Amersham Bioscience, Uppsala, Sweden). Intensity of the signals was quantified with NIH image software (National Institutes of Health, Bethesda, MD, USA). ERK1/2 activation was expressed as a ratio of phospho-ERK1/2 amount relative to the total amount of ERK1/2. Akt activation was expressed as a ratio of phospho-Akt amount relative to the total amount of Akt.

Statistical analysis

Data were expressed as mean±S.E. of values from several experiments and statistical significance was evaluated using the

unpaired *t*-test. A value of $p<0.05$ was considered statistically significant.

Results

Effects of PKA inhibitors on adipocyte differentiation of 3T3-L1 cells

We examined the effects of four kinds of PKA inhibitors, H-89, PKI, Rp-cAMP, and KT 5720, on adipocyte differentiation. H-89 and PKI are inhibitors of the catalytic subunit of PKA (Chijiwa et al., 1990; Day et al., 1989) and Rp-cAMP is an inhibitor of the regulatory subunit (Yokozaki et al., 1992). Triglyceride content in the cells treated with 10 μM H-89 was significantly increased (Fig. 1A). Oil-Red O staining showed the increased lipid droplets, although the cell count in the H-89-treated cells was not significantly different from that in the control cells (data not shown). In contrast, treatment with PKI or Rp-cAMP did not affect triglyceride content (Fig. 1A). KT 5720, another PKA inhibitor which is structurally different from H-89 (Son et al., 2006; Sun Park et al., 2006), was also tested and found to have no effect on triglyceride content (data not shown).

We further investigated the effects of the PKA inhibitors on the expression of adipocyte markers by quantitative real-time RT-PCR. We employed PPARγ and aP2 as adipocyte-specific markers, and 36B4 as a control because its expression is not influenced by the differentiation of 3T3-L1 cells (Spiegelman, 1998; Thompson et al., 2004). As shown in Fig. 1B and C, the expression of PPARγ and aP2 mRNAs in 3T3-L1 cells treated with 10 μM H-89 was significantly increased by 3.1-fold and 3.7-fold, respectively. However, the treatment with PKI or Rp-cAMP had no effect on the expression of PPARγ and aP2 mRNAs.

Then, the dose responsiveness of the enhancing effect of H-89 on adipocyte differentiation was examined and it was found that the treatment with 0.1 and 1 μM H-89 had no effect on triglyceride content in contrast to the treatment with 10 μM H-89 (Fig. 1D). The treatment with 10 μM H-85, which is an inactive form of H-89 (Son et al., 2006; Sun Park et al., 2006), enhanced triglyceride content as markedly as the treatment with H-89 (Fig. 1D).

These results indicated that H-89 promotes adipogenesis of 3T3-L1 cells independently of inhibition of PKA activity.

Effects of H-89 on insulin signaling in 3T3-L1 cells

Insulin is generally considered to play a crucial role in the process of adipogenesis (Uehara et al., 1991, 1994). To determine whether H-89 modulates insulin signaling, we examined the effect of H-89 on both pathways of Ras/ERK1/2 and PI3K/Akt, which are downstream signaling of insulin.

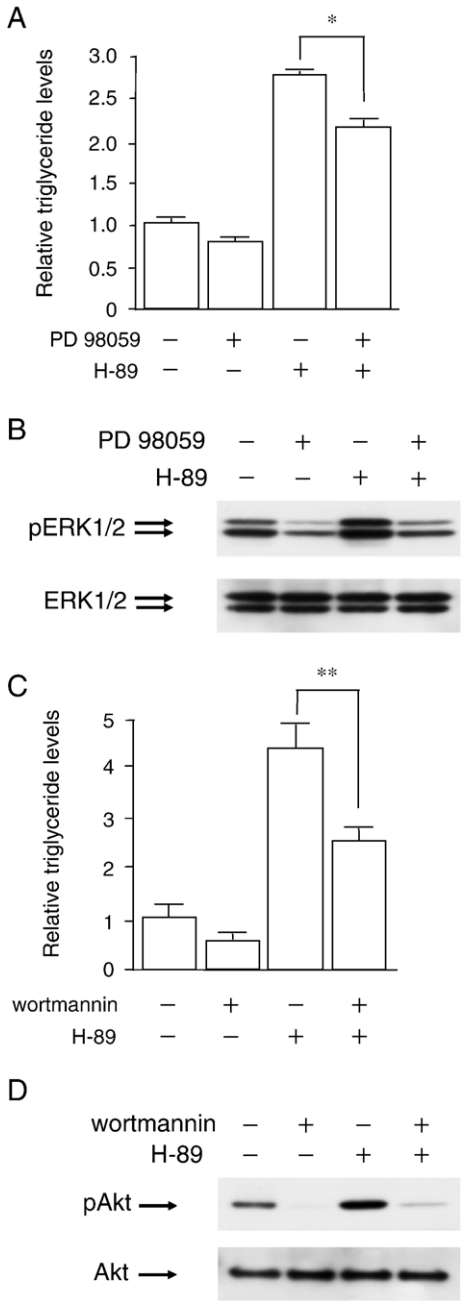

Fig. 3. Effects of MEK inhibitor PD 98059 or PI3K inhibitor wortmannin on the H-89-induced increase in triglyceride content of 3T3-L1 cells and on the H-89-induced potentiation of the phosphorylation of ERK1/2 or Akt in 3T3-L1 cells. (A) Triglyceride content in 3T3-L1 cells treated with 20 μM PD 98059 alone, 10 μM H-89 alone or the mixture of 20 μM PD 98059 and 10 μM H-89 at day 12. Cells were treated with the differentiation medium in the absence or presence of the materials to be tested for 4 days after induction. The level of triglyceride content was expressed relative to that in the cells treated in the absence of the materials to be tested. (B) Representative immunoblots of phospho-ERK1/2 and total ERK1/2. Cells were treated with the differentiation medium in the absence or presence of 20 μM PD 98059 alone, 10 μM H-89 alone or the mixture of 20 μM PD 98059 and 10 μM H-89 for 10 min after induction. Cell lysates were analyzed for phospho-ERK1/2 (pERK1/2) and total ERK1/2 (ERK1/2) by immunoblotting using specific antibodies. (C) Triglyceride content in 3T3-L1 cells treated with 1 μM wortmannin alone, 10 μM H-89 alone or the mixture of 1 μM wortmannin and 10 μM H-89 at day 12. Cells were treated with the differentiation medium in the absence or presence of the materials to be tested for 4 days after induction. The level of triglyceride content was expressed relative to that in the cells treated in the absence of the materials to be tested. (D) Representative immunoblots of phospho-Akt and total Akt. Cells were treated with 1 μM wortmannin alone, 10 μM H-89 alone or the mixture of 1 μM wortmannin and 10 μM H-89 for 10 min after induction. Cell lysates were analyzed for phospho-Akt (pAkt) and total Akt (Akt) by immunoblotting using specific antibodies. Data were obtained from 3 independent experiments ($n=6$). Values are means±S.E. * indicates $p<0.005$ and ** indicates $p<0.05$.

Approximately 2-fold increase in phospho-ERK1/2 level was found in the H-89-treated cells without apparent alteration in the total ERK1/2 protein level (Fig. 2A, B) and the similar results were observed in phospho-Akt level (Fig. 2C, D). However, the treatment with PKI or Rp-cAMP had no effect on the phosphorylation levels of both kinases (Fig. 2). These results indicated that H-89 potentiates both Ras/ERK1/2 and PI3K/Akt pathways of insulin signaling.

Effects of mitogen-activated protein kinase kinase (MEK) inhibitor or PI3K inhibitor on the H-89-induced increase in triglyceride content of 3T3-L1 cells and on the H-89-induced potentiation of phosphorylation of ERK1/2 or Akt in 3T3-L1 cell

To further determine participation of both Ras/ERK1/2 and PI3K/Akt pathways in the H-89-induced potentiation of adipogenesis, we examined the effects of MEK inhibitor PD 98059 and PI3K inhibitor wortmannin on both the H-89-induced increase in the triglyceride content and the H-89-induced potentiation of phosphorylation of ERK1/2 and Akt. The H-89-induced increase in triglyceride content was significantly suppressed in the cells treated with PD 98059 or wortmannin in contrast to the increase in the untreated cells (Fig. 3A, C). The H-89-induced increase in triglyceride content was decreased by approximately 35% in the cells treated with PD 98059 and 55% in the cells treated with wortmannin (Fig. 3A, C).The treatment with PD 98059 remarkably inhibited the H-89-induced potentiation of phosphorylation of ERK1/2 without apparent alteration in total ERK1/2 protein level (Fig. 3B). Likewise, the treatment with wortmannin remarkably inhibited the H-89-induced potentiation of phosphorylation of Akt without apparent alteration in total Akt protein level (Fig. 3D).

These results suggested that the potentiation of adipogenesis by H-89 results from the activation of both Ras/ERK1/2 and PI3K/Akt pathways of insulin signaling.

Effects of Rho kinase inhibitor on adipocyte differentiation of 3T3-L1 cells and the functional relationship between H-89 and Rho kinase inhibitor

To determine whether the inhibition of Rho/Rho kinase pathway is implicated in the H-89-induced potentiation of adipocyte differentiation, we examined the effects of Rho kinase inhibitor Y-27632, H-89, and the mixture of Y-27632 and H-89 on triglyceride content and expression of PPARγ and aP2 mRNAs. When the cells were treated with Y-27632 alone, triglyceride content was significantly increased although the extent of increase was somewhat lower than that obtained by the treatment with H-89 (Fig. 4A). The mixture of Y-27632 and H-89 significantly increased triglyceride content. However, the extent of increase did not exceed that obtained by H-89 alone (Fig. 4A).

Essentially the same results were obtained in the experiments to investigate the effects of Y-27632, H-89, the mixture of both agents on the expression of PPARγ and aP2 mRNAs. Y-27632 increased the expression of PPARγ and aP2 mRNAs to a somewhat lower extent than that obtained by H-89 (Fig. 4B, C).

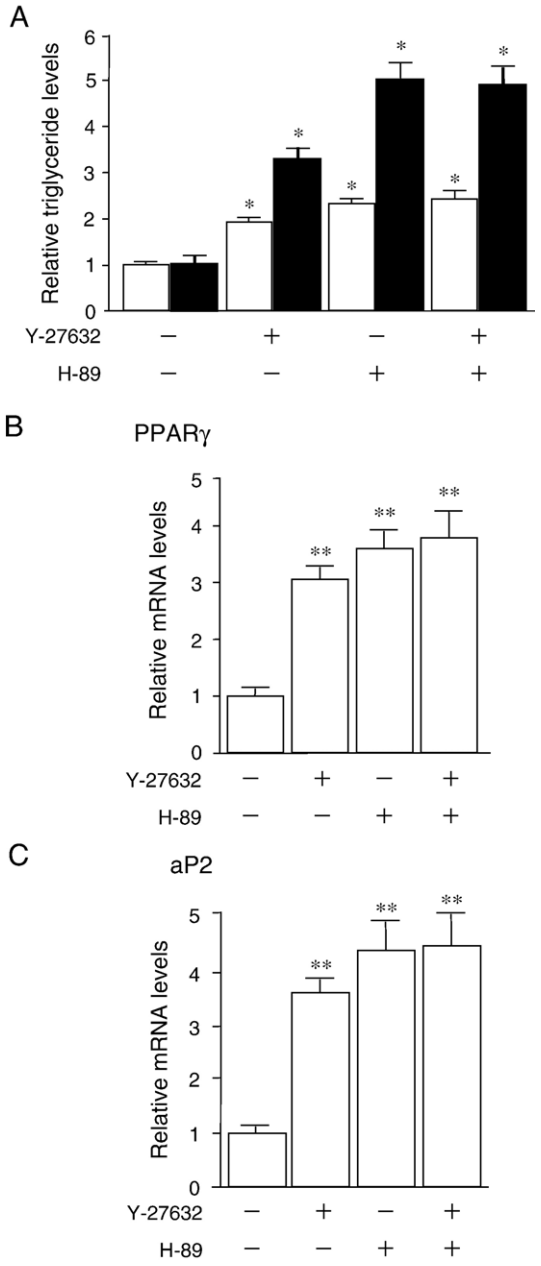

Fig. 4. Effects of Rho kinase inhibitor Y-27632 or the mixture of Y-27632 and H-89 on adipocyte differentiation of 3T3-L1 cells. (A) Triglyceride content in 3T3-L1 cells treated with 10 μM Y-27632, 10 μM H-89 or the mixture of 10 μM Y-27632 and 10 μM H-89 at day 6 (open column) and day 12 (solid column). Cells were treated with the differentiation medium in the absence or presence of the materials to be tested for 4 days after induction. The level of triglyceride content was expressed relative to that in the cells treated in the absence of the materials to be tested. Effects of Y-27632 or the mixture of Y-27632 and H-89 on the expressions of PPARγ mRNA (B) and aP2 mRNA (C). 36B4 mRNA was used as internal control. Total RNA was extracted from 3T3-L1 cells using the Trizol reagent. Three micrograms of total RNA was reverse-transcribed using Transcriptor Reverse Transcriptase. The mRNA level was expressed relative to that in the cells treated in the absence of the materials to be tested. Data were obtained from 3 independent experiments (n=7–9). Values are means±S.E. * indicates $p<0.001$ and ** indicates $p<0.005$. The difference between the effect of Y-27632 and that of H-89 on triglyceride content at day 6 and day 12 is significant ($p<0.05$).

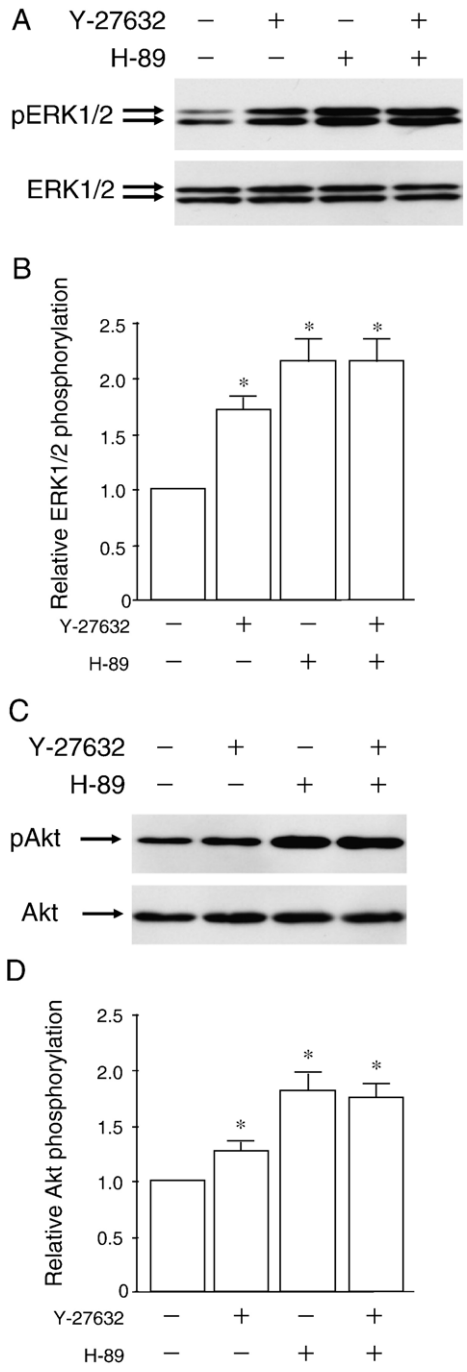

Fig. 5. Effects of Y-27632 or the mixture of Y-27632 and H-89 on insulin signaling. (A) Representative immunoblots of phospho-ERK1/2 and total ERK1/2. Cells were treated with the differentiation medium in the absence or presence of 10 μM Y-27632, 10 μM H-89 or the mixture of 10 μM Y-27632 and 10 μM H-89 for 10 min after induction. Cell lysates were analyzed for phospho-ERK1/2 (pERK1/2) and total ERK1/2 (ERK1/2) by immunoblotting using specific antibodies. (B) Relative ratio of phospho-ERK1/2 to total ERK1/2. The level of ERK1/2 phosphorylation was expressed relative to that in the cells treated in the absence of the materials to be tested. (C) Representative immunoblots of phospho-Akt and total Akt. Cell lysates were analyzed for phospho-Akt (pAkt) and total Akt (Akt) by immunoblotting using specific antibodies. (D) Relative ratio of phospho-Akt to total Akt. The level of Akt phosphorylation was expressed relative to that in the cells treated in the absence of the materials to be tested. Data were obtained from 3 independent experiments. Values are means ± S.E. * indicates $p<0.05$. The difference between the effect of Y-27632 and that of H-89 on the phosphorylation of Akt and ERK1/2 is significant ($p<0.05$).

The effect of the mixture of both agents on the expression of these mRNAs did not exceed the effect of H-89 alone (Fig. 4B, C).

Furthermore, Y-27632 increased the phosphorylation levels of ERK1/2 and Akt by approximately 1.7-fold and 1.3-fold, respectively (Fig. 5). H-89 increased the phosphorylation levels of ERK1/2 and Akt by approximately 2.2-fold and 1.9-fold, respectively (Fig. 5). Similarly to the findings on triglyceride content and the expression of adipocyte markers, the mixture of Y-27632 and H-89 did not exhibit additive enhancing effects on the phosphorylation of both kinases (Fig. 5). These results suggested that H-89 has a very similar action to that of the Rho kinase inhibitor and the suppression of Rho/Rho kinase by H-89 is involved in the activation of insulin signaling that eventually leads to the potentiation of adipocyte differentiation.

Discussion

In the present study, we observed that H-89 remarkably enhanced adipocyte differentiation of 3T3-L1 cells, whereas the other three PKA inhibitors, PKI, Rp-cAMP, and KT 5720, did not enhance adipocyte differentiation. Moreover, H-89 remarkably enhanced phosphorylation of both Akt and ERK1/2, but the other two PKA inhibitors, PKI and Rp-cAMP did not enhance their phosphorylation. It was also found that rather a high concentration of H-89 such as 10 μM was needed to enhance adipocyte differentiation in comparison with the concentrations of H-89 to block PKA activity (Chijiwa et al., 1990; Davies et al., 2000; Konda et al., 1994), and that H-85, which is an inactive form of H-89 (Son et al., 2006; Sun Park et al., 2006), had a similar enhancing effect on adipocyte differentiation. These results suggested that H-89 promotes adipogenesis of 3T3-L1 cells independently of inhibition of PKA activity.

It is possible that insulin/IGF-1 signaling can activate two kinds of kinase pathways, Ras/ERK1/2 and PI3K/Akt pathways. Akt is an important signal mediator in IGF-1 receptor signal cascade involved in the induction of adipocyte differentiation (Xu and Liao, 2004). For example, inhibition of PI3K with wortmannin blocks the adipocyte differentiation of 3T3-L1 cells (Tomiyama et al., 1995). The ectopic expression of activated Akt induces the differentiation of 3T3-L1 preadipocytes into adipocytes (Kohn et al., 1996; Magun et al., 1996). In contrast, the role of Ras/ERK1/2 signaling in adipogenesis has yet to be clarified. Some studies asserted that activation of mitogen-activated protein kinase (MAPK) by various effectors blocks adipogenesis (Font de Mora et al., 1997; Kim et al., 2001), whereas others claimed that it promotes adipocyte differentiation (Prusty et al., 2002; Xu and Liao, 2004; Zhang et al., 1996). Recently, analysis of ERK1 (−/−) mice indicated that ERK1 is implicated in the regulation of adipocyte differentiation (Bost et al., 2005). In our experiments, MEK inhibitor as well as PI3K inhibitor suppressed both the H-89-induced increase in triglyceride content of 3T3-L1 cells and the H-89-induced potentiation of phosphorylation of ERK1/2 and Akt. These observations suggested that H-89 activates not only the PI3K/Akt pathway but also the MAPK pathway, thereby promoting adipogenesis.

Further, we showed that the Rho kinase inhibitor Y-27632 enhanced the phosphorylation of Akt and ERK1/2 and thereby promoted adipogenesis, although Y-27632 were somewhat less effective than H-89. It is generally considered that the activation of Rho-dependent kinase-α phosphorylates the serine residue of IRS-1 and consequently results in the inhibition of the following processes: (1) IRS-1 tyrosine phosphorylation, (2) association of p85 subunit of PI3K with IRS-1 and (3) insulin stimulation of the PI3K/Akt pathway (Begum et al., 2002). In a recent study, activated Rho kinase was found to increase the serine phosphorylation of IRS-1 and thereby downmodulate insulin/IGF-1 signaling, leading to the inhibition of adipocyte differentiation and promotion of myocyte differentiation (Sordella et al., 2003). In contrast, the cross-talk between Rho/Rho kinase and Ras/ERK1/2 pathways has not been completely understood, although there was a report that the activation of Rho/Rho kinase pathway was essential for stretch-induced ERK activation in mesangial cells (Krepinsky et al., 2003). Taking these findings into consideration, our results relating to the Rho kinase inhibitor Y-27632 suggested that the suppression of Rho/Rho kinase pathway is implicated in the activation of not only the PI3K/Akt pathway but also the MAPK pathway.

Earlier reports demonstrated that H-89 inhibits several protein kinases including Rho kinase with potency similar to or greater than the action against PKA (Davies et al., 2000; Leemhuis et al., 2002). We revealed that the mixture of H-89 and Y-27632 did not exhibit additive enhancing effects on either adipogenesis or phosphorylation of both kinases. These results suggested that the major possible mechanism whereby H-89 potentiates adipocyte differentiation is the activation of insulin signaling that is elicited mostly by inhibiting Rho/Rho kinase pathway.

There have been reports which show that H-89 directly inhibit ion channels, such as KATP channels and Kv1.3 channels independently of PKA (Son et al., 2006; Sun Park et al., 2006), and that these ion channels are involved in glucose uptake (Li et al., 2006; Miki et al., 2002). At present, however, the correlation of the enhancing effect of H-89 on adipogenesis with its inhibitory effect on ion channels is not clear.

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